UNIVERSITY OF MICHIGAN
JAN 17 1955
MEDICAL LIBRARY

# The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 45 JANUARY, 1955 NO. 1

*The Official Publication of the Central Society for Clinical Research*

Letter from the Past Editors ---- 1

Presidential Address. Jerome W. Conn, M.D., Ann Arbor, Mich. ---- 3

Clinical and Experimental

Thrombocytopenic Purpura Caused by Hypersensitivity to Quinine. Ruth Steinkamp, M.D., Carl V. Moore, M.D., and William G. Doubek, M.D., St. Louis, Mo. ---- 18

The Hemolytic Effect of Primaquine. V. Primaquine-Sensitivity as a Manifestation of a Multiple Drug Sensitivity. Raymond J. Dern, M.D., Ph.D., Ernest Beutler, M.D., and Alf S. Alving, M.D., Chicago, Ill. ---- 30

The Hemolytic Effect of Primaquine. VI. An In Vitro Test for Sensitivity of Erythrocytes to Primaquine. Ernest Beutler, M.D., Raymond J. Dern, M.D., Ph.D., and Alf S. Alving, M.D., Chicago, Ill. ---- 40

The Persistence in the Blood of the Radioactive Label of Albumins, Gamma Globulins, and Globulins of Intermediate Mobility. II. Comparison of Gamma Globulins Labeled with $S^{35}$ and $I^{131}$ in the Same Subjects. S. H. Armstrong, Jr., M.D., John Kukral, M.S., M.D., Jerome Hershman, M.S., Katharine McLeod, M.S., Janet Wolter, M.D., and David Bronsky, M.D., Chicago, Ill. ---- 51

Changes in Protein Fractions of Dog Plasma After Bleeding and Dextran Infusion. Robert E. Semple, M.A., Ph.D., Kingston, Ontario ---- 61

Animal Distribution of Colloids of $Au^{198}$, $P^{32}$, and $Y^{90}$: An Improved Method of Tissue Assay for Radioactivity. T. N. Lahr, M.S., Robert Olsen, A.B., G. I. Gleason, B.Ch.E., and D. L. Tabern, Ph.D., North Chicago, Ill. ---- 66

The Immunologic Response of Mice From Stocks Resistant and Susceptible to Acute Disseminated Encephalomyelitis. Peter K. Olitsky, M.D., and Johanna M. Lee, Ph.D., New York, N. Y. With the Technical Assistance of Sophie L. Skadorwa, A.B. ---- 81

The Effect of Antibiotics on the Excretion of Phenolic Compounds. Walter F. Rogers, Jr., M.D., Mary P. Burdick, B.S., and George R. Burnett, M.D., Syracuse, N. Y. ---- 87

In Vitro Activity of Chloramphenicol on Salmonella Typhi. Kenneth C. Watson, M.B., Ch.B. (Aber.), Pietermaritzburg, South Africa ---- 97

Effect of Acute Renal Failure on the Plasma Volume and Volume of Packed Red Cells in the Dog. Harold Brown, M.D., A. A. Iliya, M.D., P. B. Price, M.D., and O. N. Rambo, Jr., M.D., Salt Lake City, Utah ---- 102

*(Contents continued on inside front cover)*

PUBLISHED BY THE C. V. MOSBY COMPANY, ST. LOUIS 3, MISSOURI

Copyright 1955 by The C. V. Mosby Company

Contents—*(Continued from front cover)*

Body Composition and Oxygen Consumption of Overweight, Normal, and Underweight Women. Louis C. Johnston, M.D., and Lionel M. Bernstein, M.D., Chicago, Ill. 109

The Nonprotein Soluble Sulfhydryl Content of Human Leukocytes and Erythrocytes in Infection and Leukemia. Rosalie Green and Samuel P. Martin, M.D., Durham, N. C. 119

Properties of Plasma Thromboplastin Antecedent (PTA) In Relation to Blood Coagulation. Robert L. Rosenthal, M.D., New York, N. Y. With the Technical Assistance of Esther Gendelman, B.S. 123

Perfusion Studies of the Isolated Young Rat Liver. David L. Gunberg, Ph.D., William R. Lyons, M.D., Ph.D., and Ruth E. Johnson, A.B., Berkeley, Calif. 130

In Vitro Studies of Red Cell Fragility in Anti-D Hemolytic Disease of the Newborn. Tibor J. Greenwalt, M.D., and Demetrios C. Triantaphyllopoulos, M.D., Milwaukee, Wis. With the Technical Assistance of Mavis Moorman 135

## Laboratory Methods

An "Anti-Infection Cannula" for Syringes to Prevent Transmission of Infections in Mass Immunization Campaigns. K. H. Gan, M.D., Djakarta, Indonesia. With the Technical Assistance of Tjioe Swie Bie and R. Soekardi Atmadja 141

Correction of Paper-Strip Serum Electrophoresis Diagrams. A. L. Latner, M.Sc., M.D., D.I.C., F.R.I.C., Newcastle Upon Tyne, England 147

The Manometric Analysis of Respiratory Gases in Blood Containing Volatile Anesthetic Agents. I. A Comparison of Macromethods. Duncan A. Holaday, M.D., and Mariagnes Verosky, B.A., New York, N. Y. 149

Improved Resistance Wire Strain-Gauge Manometers Adaptable for Biologic Measurements. Earl H. Wood, M.D., Ph.D., and William Sutterer, B.S., Rochester, Minn. 153

A Rapid Procedure for the Determination of Lead in Blood or Urine in the Presence of Organic Chelating Agents. Samuel P. Bessman, M.D., and Ennis C. Layne, Jr., M.S., Washington, D. C. 159

Editor

**WILLIAM D. ROBINSON, M.D.**

Associate Editor

**WILLIAM H. BEIERWALTES, M.D.**

University Hospital
University of Michigan Medical School
1313 Ann Street, Ann Arbor, Michigan

## Board of Editors

S. HOWARD ARMSTRONG, JR., M.D.
University of Illinois, Chicago

FRANK H. BETHELL, M.D.
University of Michigan, Ann Arbor

RICHARD B. CAPPS, M.D.
Northwestern University Medical School, Chicago

A. C. CORCORAN, M.D.
Cleveland Clinic, Cleveland

ELMER L. DeGOWIN, M.D.
State University of Iowa, Iowa City

RICHARD V. EBERT, M.D.
University of Arkansas, Little Rock

WENDELL H. HALL, M.D.
University of Minnesota, Minneapolis

LEON O. JACOBSON, M.D.
University of Chicago, Chicago

OLIVER H. LOWRY, M.D.
Washington University, St. Louis

W. A. SODEMAN, M.D.
University of Missouri, Columbia

RANDALL G. SPRAGUE, M.D.
Mayo Clinic, Rochester

RICHARD W. VILTER, M.D.
University of Cincinnati, Cincinnati

Vol. 45, No. 1, January, 1955. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription price: United States and its Possessions $12.00 per year; Canada $12.50; Foreign $13.50. Subscriptions to medical students, interns, and residents, half-price. Entered as Second Class Matter October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

Medical

# The Journal of Laboratory and Clinical Medicine

VOL. 45 JANUARY, 1955 No. 1

## LETTER FROM THE PAST EDITORS

AS OF January 1, Dr. William D. Robinson, Professor of Medicine at the University of Michigan School of Medicine, became Editor of the JOURNAL OF LABORATORY AND CLINICAL MEDICINE. He has chosen Dr. William H. Beierwaltes, Associate Professor of Medicine, as his Associate Editor. Drs. E. M. K. Geiling, T. H. Hunter, and Eugene B. Ferris have completed, respectively, six-, four-, and three-year terms on the Board. On behalf of the Central Society for Clinical Research and the Editorial Board we wish to express our appreciation for their valuable services. Dr. Geiling will not be replaced on the Board which will reduce the membership to twelve. Dr. W. A. Sodeman, Professor of Medicine, University of Missouri, has accepted a three-year term on the Board.

During the past six years the Editors and Board members have called upon many of their associates and colleagues in other universities to aid them in the evaluation of manuscripts. Those who have participated in this capacity during the past year are:

Achor, Leonard B.
Adams, Wright
Barron, E. S. G.
Beierwaltes, William H.
Benditt, Earl P.
Benham, Ross S.
Bergenstal, Delbert M.
Bethard, William F.
Bird, O. D.
Brozek, Joseph
Burch, George E.
Cannon, Paul R.
Cartwright, G. E.
Clark, Dwight E.
Conn, Jerome W.
Daughaday, William H.
Davidsohn, Israel
Dorfman, Albert
DuBois, Kenneth P.
Ebert, Richard V.
Foreman, Harry
Freeman, Smith
Furth, Jacob
Goldwasser, Eugene
Grayston, J. Thomas
Harrington, William
Hasterlik, Robert J.
Heller, Ben I.
Huff, Clay G.
Humphreys, Eleanor M.
Jones, Richard J.
Katy, Seymour S.
Katz, Louis N.
Keating, F. R., Jr.
Kirsner, Joseph B.
Koch-Weser, Dieter
Landau, Richard L.
Lepper, Mark H.
LeRoy, George V.
Lorincz, Allan L.
Mandlowitz, Samuel
Martin, Frank E.
Martin, Samuel P.
Mason, Harold L.
Mathews, Martin
Mattox, Vernon R.
Miller, C. Phillip
Moore, Carl V.
Neel, James
Okita, George T.
Pick, R.
Pollard, H. M.
Ponder, Eric
Procknow, John J.
Pullman, Theodore N.
Putnam, Frank W.
Quick, Armand J.
Rammelkamp, Charles H.
Rhead, Clifton C.
Robinson, William D.
Rosenberg, Edward F.
Rossing, Robert
Roth, Lloyd J.

Rothman, Stephen
Scott, T. F. McNair
Seal, John R.
Smith, David T.
Snapper, I.
Spink, Wesley W.
Spurling, Carrol L.
Stead, William W.
Talmage, David W.
Thomas, Lewis
Turner, Thomas B.
Watson, Cecil J.
Weinstein, Irwin M.
West, Clark
Wilson, Joseph S.
Witebsky, Ernest
Wood, Earl H.

We wish to take this opportunity to thank the past and present members of the Editorial Board for their excellent service to the JOURNAL and the Central Society for Clinical Research. We would also like to express our appreciation for the cooperation we have received from The Mosby Company.

Drs. Block, Ebert, and I have considered it an honor and privilege to serve the Central Society for Clinical Research and The Mosby Company as Editors of this JOURNAL and we extend to the new Editors, Drs. Robinson and Beierwaltes, our congratulations and best wishes.

CLAYTON G. LOOSLI, M.D.
Editor
MATTHEW BLOCK, M.D.
ROBERT H. EBERT, M.D.
Associate Editors

# PRESIDENTIAL ADDRESS

## PART I. PAINTING BACKGROUND

## PART II. PRIMARY ALDOSTERONISM, A NEW CLINICAL SYNDROME*

JEROME W. CONN, M.D.
ANN ARBOR, MICH.

### PART I. PAINTING BACKGROUND

AT THIS time in our annual meeting your President, having been duly elected unanimously in accordance with prevailing democratic practices, is called upon to justify his presidential existence. It is my fondest hope that each and every one of you will some day be similarily elected by acclamation, for, as all who have been in this spot before me will attest, it is a unique experience indeed. One important aspect of this job is that it affords an unfailing opportunity to catch up on past and current philosophy regarding clinical investigation and clinical investigators. As of this morning, I am steeped in presidential addresses. In this field I can quote the literature with uncanny accuracy and with a very fine degree of discrimination. For anyone who may feel the need for consultation in this regard, my services will be available for a very limited period of time.

Let me recapitulate in an entirely different vein and with absolute sincerity. To have been honored by election to the presidency of a Society which has been an important and integral part of my professional life, and which has been an inspiration toward the goal of soundness in clinical research, has produced in me an even more intense feeling of closeness to it and appreciation of it. Had I paused previously to think about the Society as seriously as I have during the past year, I would have derived the same benefits without the high office. A little serious thinking by each one of our members will bring to him forcefully the total meaning of a Society such as ours. Develop this meaning and then guard it jealously, for you will be forced to conclude that this Society is a forum from which is demonstrated the stimulus, the spirit, the principle, and the practice of free enterprise in our field of research.

In recent years our membership has constituted a relatively small proportion of the total number of observers at our annual meeting. Our guests represent a superlative segment of the medical profession. Why do they come to our meetings, many of them year after year? It is not because they aspire to membership, for attendance at meetings is not a prerequisite; nor do they anticipate world-shaking discoveries which can be applied quickly in their daily work. They come because they have discovered a stimulating experience. They are sharing with us the thrills of watching sharp, competent, and

From the Metabolic Research Unit, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Mich.

Presented at the Twenty-seventh Annual Meeting of the Central Society for Clinical Research, Chicago, Ill., Oct. 29, 1954.

*Supported by the Research and Development Board, Office of the Surgeon General, U.S. Army.

eager young minds struggle to make sense in that twilight zone between light and darkness. Let us be consciously aware of what it is that appeals to our minds and brings us together with enthusiasm for each succeeding meeting. For then our attitudes and demeanor will be oriented properly.

Despite my flippant comments regarding presidential addresses, I must admit that I found many of them provocative and an occasional one definitely inspiring. I have prepared no comprehensive review of my personal philosophy of clinical investigation. Instead, I plan to make a scientific report to you about a clinical syndrome, the investigation of which has been most exciting to me since I initiated it in April of this year. I do this despite the warning voiced in one of our recent presidential addresses that "Frustrated investigators have even used this opportunity (the President's address) to give a paper which former incumbents of the office had omitted from previous programs."[1]

But before placing these data before you, I do wish to express a few simple and homely thoughts about us and our meetings which have seemed important to me. Colleagues from my own laboratory will recognize these feelings, even though, today, the latter are dressed up in their society clothes.

Interestingly enough, I find that the swan songs of Society presidents rarely mention anything about the Society. This may be related to the fact that "state of the union" messages are sometimes distasteful, or to the fact that the elected officers are expected to guide the destinies of the Society with the least possible mental inconvenience to the membership at large. While your Council appreciates this perennial vote of confidence, it should like to urge a show of interest by the membership in the form of letters to the Council whenever the former feels that the Society stands to benefit by a suggestion. Only in this way can the Council know whether or not the Society is serving all of the purposes for which it was established.

This Society was born in a spirit of helpfulness to young investigators. It was believed that it would stimulate original work and at the same time give young investigators an opportunity to present to their colleagues the results of their labors. I am certain that to date these objectives have been very well fulfilled. I am disturbed, however, by a very subtle and gradual change in the *personality* of this Society. Much of this change has occurred during the past ten years, a period of most rapid growth for our Society. It may be inevitable that personalities must change with increasing age and size, but if our objective remains the same, namely, stimulation and helpfulness to young investigators, our attitudes and perspectives must not be allowed to change to a point at which our objectives can be endangered.

An air of friendly informality has been one of the most pleasing attributes of this particular scientific Society. I do not wish it to be said in the future that an effort was not made to preserve it, for it constitutes the essence of helpfulness and encouragement to the young investigator.

I have noted a wave of resentment and embarrassment to pass through this hall when the discussor of a paper has been unkind, impolite, or arrogant.

Obviously, the Society as a whole does not wish to accept such behavior, but seldom does the feeling crystallize sufficiently to be made a subject of Society business. To me, as well as to the welfare of this Society, it is a subject of utmost importance. Having been sensitized, I have observed such outbursts to occur with increasing frequency in recent years. Such an attitude on the part of members of this Society is out of tune with its objectives, namely, encouragement and helpfulness to the young investigator. Instead, the recipient of such criticism is dismayed, discouraged, disillusioned, bewildered, and occasionally completely disoriented. From *his* experience, many of his young colleagues in the listening audience register a distorted picture of the true purpose of our Society.

Admittedly each individual sees things in his own image and his reactions are influenced largely by his own past experiences, but for purposes of reassurance to our young colleagues I wish to describe the emotional state of the young man who is about to present a paper before our Society, and to indicate how *I* think we and he should view this situation. If he is not downright frightened he is at least apprehensive. A glance at the knees from where the chairman sits affords ample confirmatory evidence. But why is he so disturbed? To be sure he is required to say his piece and be done in ten minutes. But this is no cause for concern, because he has practiced giving his paper and is confident that nine and a half minutes is sufficient, providing nothing goes wrong with the lights, the lantern, or the operator. His *apprehension* relates to the two- or three-minute period which follows *completion* of his presentation. How will the results of his efforts be received by this austere, well-informed, and highly critical audience? Might his work be ripped to shreds on the basis of an obvious point, which had failed to occur to him? Will his knowledge of the subject and its background stand him in good stead at a minute's notice, or will he think of the correct answer during the middle of the next paper? But most of all, he hopes that whatever criticism is offered will be friendly and kindly criticism, as it was at home during his last practice session, before his departmental journal club. In any case, such thoughts represent the basis of his fear and trembling.

Older members will assure him that perspectives change with time and experience, and that relative confidence will eventually replace relative insecurity, but such assurances fail to inhibit the alarm reaction. There *are* some things, however, that can help right now. I am not old enough to have forgotten completely the perspective of our younger colleagues. Their aspirations are pointed in your direction. They wish eventually to reach the standing and respect in clinical research which they believe you have achieved, and many of them will, and perhaps to a greater degree! Let us set for them a proper example of kindness, friendliness, and common decency. Rest assured that such an attitude will not stifle constructive criticism.

And to each of the younger group may I suggest that from the time of early preparation of your essay to the time of its presentation your attitude be conditioned by the following knowledge. From among a large group of submitted abstracts, the Council, which represents the entire membership, has

*invited* you to present the results of your efforts to the Society. The Society is cognizant of the extra effort involved in preparing a concise presentation of your work and appreciates your willingness to come before the Society with it. The main point here is that *you* are not indebted to the Society for selecting your paper. The Society is grateful to you for presenting it whether you are a member or an invited guest. This being the case, you should expect, and the Society *must display, politeness.*

One final comment on attitude and conduct in this Society. The rapid growth of clinical investigation in recent years and the increased competition among investigators for recognition in their respective fields have tended to distort the true purposes and value of all such societies. This meeting must not be regarded as a personalized, competitive market place. It is a forum where new findings and new ideas are presented for the first time with the hope that they will receive serious, critical, and helpful evaluation. Recognition is a by-product of past events. Its real significance is that it implies confidence and hopefulness for future ones. It does not imply greatness or impart the privilege of an overbearing attitude toward others.

We speak glibly these days of "fundamental research" and extol the discovery of a fact, however disjointed from all other facts, as an addition to the sum total of knowledge acquired by mankind. Reason tells us that we must acquiesce in a vague kind of way to the statement that every fact has a potential usefulness. In the meanwhile it is regarded as *background,* available to all who choose to use it. Are there *any* of us capable of evaluating which fact, when eventually correlated with others, will have the greatest impact upon the lives of men? The answer is "No."

Let us remember that we are *all* painting background. Regardless of how important or unimportant your contribution of today may seem, no sooner has it been expounded than it has become background. There is some solace in the fact that your brain child is not dead! The entire background is seething with life and motion. But acceptance of the idea of painting background is sufficient to remove the undesirable gusts of wind from many sails. Let us rejoice in the knowledge that to us has come the opportuunity to paint background.

While all of these considerations are self-evident, it is my firm conviction, based upon observation in these meetings and in others of similar purpose, that they must be voiced periodically if we are to maintain the spirit which is responsible for the very existence of such societies. How are we to engender the proper spirit? That is difficult, for it involves good sportsmanship and good fellowship. Personal contact among members has changed inversely with the size of the Society. A very small step in the right direction, however, has been taken by your Council this year.

## PART II. PRIMARY ALDOSTERONISM, A NEW CLINICAL SYNDROME

And now, for a short period, I wish to paint background. This presentation I have entitled, "Primary Aldosteronism, a New Clinical Syndrome." It will be of interest that I was responsible for selecting the paper by Doctors

Mader and Iseri[2] and for having it presented just prior to this one. I recognized in their abstract that they were dealing with a variation of the abnormal mechanism which I shall describe. Fortunately, the Council did not question my motive, and I was able to maintain the usual mystery with which the substance of the President's address has traditionally been encompassed.

Descriptions of two more cases, which I believe to be a later stage of "primary aldosteronism," can be found in the recent literature.[3, 4] The present study attempts to relate a characteristic clinical picture and its associated biochemical lesion to the chronic activity of excessive amounts of a sodium-retaining steroid, presumably aldosterone.

My intense interest in the phenomena to be described stems from work carried out in my laboratory from 1943 to 1948. Those investigations had to do with different degrees of sodium depletion in normal men under conditions of intense and prolonged sweating while on low levels of sodium intake. In summarizing this work in 1949, we stated[5]: "In chronic stress of the type employed in these experiments, evidence of *continuously* increased activity of salt-saving steroids is clear. Metabolic evidence of increased activity of those steroids which affect protein metabolism is obvious initially, but gradually disappears." "Perhaps corticotropic stimulation of the adrenals can evoke a preponderant secretion of one type of steroid, depending upon the conditions, type, and duration of the stress." In 1950 we said[6]: "Changes in the concentration of sodium and chloride of thermal sweat, collected under standard conditions, parallels changes in the activity of the adrenal cortex with respect to its elaboration of steroids which act like desoxycorticosterone. The ability to observe changes in activity of salt-active corticoids is of potential value in investigative medicine, in elucidating the possible role of the salt-active corticoids in a wide variety of clinical conditions, nephrosis, congestive heart failure, essential hypertension, premenstrual edema."

Since that time an extremely potent salt-retaining steroid, now called aldosterone, has been shown to be a normal adrenal secretory product.[7] As you have already heard, this substance has been found in abnormally high concentration in the urine of edematous nephrotics, and in patients in cardiac failure or decompensated hepatic cirrhosis.[8-11] Other than the fact that all have edema, the common factor responsible for the high urinary excretions of the sodium-retaining corticoid remains unknown to date. It is obvious, however, that in these conditions the primary difficulty is not one of increased activity of sodium-retaining steroid. However the latter comes about, it is secondary to some metabolic event which is common to all three conditions in the course of their development. Such conditions I would classify, temporarily, as being associated with *secondary aldosteronism.*

Primary aldosteronism is not associated with edema, and in its pure state manifests itself in the form of an interesting complex of symptoms and a fascinating abnormality of electrolyte metabolism.

Table I gives briefly the essential clinical story of patient M. W., the subject of this investigation.

TABLE I. M. W., FEMALE, 34 YEARS OF AGE. ADMITTED TO METABOLIC RESEARCH LABORATORY, APRIL 27, 1954.

| | |
|---|---|
| Chronic complaint | Intermittent spasms, weakness and paralysis of muscles for 7 years. |
| Patient interview | October, 1947, "paralyzed" from hips down for 2 days. Several similar attacks since but less severe and shorter. Frequent attacks of great muscular weakness. Other attacks of typical tetany involving upper and sometimes lower extremities. Well between attacks. Blood pressure known to be 180 to 190 for 4 years. Trace of urinary albumin repeatedly. Polyuria and nocturia for years. No vomiting, diarrhea, or drugs |
| Physical examination | Well-developed musculature. Positive Chvostek and Trousseau signs. Blood pressure 176/104. Heart size normal. No edema. Reflexes hyperactive |

TABLE II. INITIAL LABORATORY VALUES, M. W.

| BLOOD VALUES | | URINARY AND RENAL VALUES |
|---|---|---|
| 1. Counts normal, Hb | 14.4 Gm. | 1. Urine protein O to Tr., Sed. neg. |
| 2. Eosin | 198/c. mm. | 2. pH 7.17-7.54 |
| 3. Serum creatinine | 0.54-1.02 | 3. End. Creat. Clearance 125-170 L./day |
| 4. NPN | 33-38 mg. % | 4. PSP 50% in 15 min., 80% in 2 hr. |
| 5. Sugar | 84 mg. % | 5. 18 h conc. test sp. gr. 1.011 |
| 6. Serum Protein | 6.9%, alb. 4.9% | 18th h 10 mg. pitressin. 20th h sp. gr. 1.011 |
| 7. Serum Ca | 10.2 mg. % | 6. Ca 122 mg./day with intake 137 mg./day |
| 8. Serum Inorg. P. | 2.4 mg. % | 7. 17-ketosteroids 5.7 mg./day |
| 9. Serum Cl | 101-103 meq./L. | 8. 17-hydroxysteroids 5.0 mg./day |
| 10. Serum Na | 146-151 | 9. K-U/P 13 at serum K-2.5 meq. |
| 11. Serum K | 1.6-2.5 | Metabolic alkalosis with hypokalemia and tetany |
| 12. Serum pH | 7.62 | *Possibilities* |
| 13. $CO_2$ comb. power | 82 vol. % | 1. Excessive activity of aldosterone |
| | | 2. Specific renal tubular lesion with K loss |

Program: To have complete balance study with assays for aldosterone

Table II shows the base-line laboratory values. The striking abnormalities in the blood are the hypokalemia, hypernatremia, and alkalosis. It is to be noted that urinary 17-ketosteroid and 17-hydroxysteroid*[12] excretions are normal. The renal function studies indicate reasonably normal glomerular and tubular function, except for the presence of a small amount of proteinuria intermittently and a low, fixed urinary specific gravity. The excellent study by Schwartz and Relman[13] proved that in uncomplicated hypokalemia, due to diarrhea, urinary specific gravity was fixed at very low levels, and that renal concentrating ability returned to normal four to ten weeks after repletion of the body with potassium. It is noteworthy further that the test with pitressin would indicate that the low specific gravity is not due to secondary depression of antidiuretic hormone. A battery of liver function tests gave no suggestion of any hepatic impairment.

On the basis of these initial data we suggested that this abnormality might be due to excessive activity of aldosterone, or that it might represent what had been described before as a specific renal tubular lesion resulting in potassium loss.[3, 4]

Table III shows the patient's assays for sodium-retaining corticoid as compared with those done on seven normal people. Our normal values agree

*We wish to express our gratitude to Dr. R. H. Silber, Merck Institute for Therapeutic Research, for allowing us to use his excellent method for eight months prior to its publication.

TABLE III. BIOASSAYS OF URINARY SODIUM-RETAINING CORTICOID

| *Sex* | *Age* | *Sodium Output Bioassay* μg. DCA/day | |
|---|---|---|---|
| | *Normal Adults* | | |
| F | 27 | 115 | |
| F | 28 | 82 | |
| F | 39 | * | Minus value |
| F | 23 | 29 | |
| M | 32 | 24 | Range by Luetscher, 1954 |
| M | 19 | 101 | (75-113) |
| M | 19 | 79 | |
| *Patient M. W., F, Age 34* | | | |
| (8 assays on 5 consecutive urine samples) | | | |
| | No. 1 | 2,300 | *No Acid Hydrolysis* |
| | No. 2 | 1,300 | 600 |
| | No. 3 | 300 | |
| | No. 4 | 1,700 | |
| | No. 4 | 1,700 | (Repeat) |
| | No. 5 | 700 | 500 |

Method of Luetscher, but using acid hydrolysis of urine at pH 1.0.
*Means effect opposite from DCA, i.e., increased sodium output.

reasonably well with those reported by Luetscher[14] last month for normal adults. The values obtained in M. W. are so greatly elevated that there can be little doubt that her urine contains too much sodium-retaining corticoid.

Fig. 1 shows the electrolyte composition of sweat and of saliva during thirty-five days of consecutive sampling. It is to be noted, first, that throughout the entire study, and regardless of what experiment was carried out, the sweat and saliva values for sodium and chloride were very greatly depressed from normal figures, while the potassium concentrations in both instances are abnormally high. Thus, with abnormally high values for serum sodium, the concentrations of sodium in sweat and saliva are too low; and with very low serum values for potassium, the concentrations of potassium in sweat and saliva are too high. ACTH appeared to intensify mildly these changes, but the mechanism in the sweat and salivary glands which reflects activity of sodium-retaining corticoid is going so hard already, that large changes are virtually impossible. Administration of potassium, however, further lowered the concentration of sodium in sweat and saliva. This suggests that potassium loading in such a patient simply activates the abnormal mechanism further. In other words, the body resists potassium repletion.

In this connection I should mention that between two periods of study the patient ingested 180 meq. of potassium daily for twenty-nine days in addition to that contained in a normal diet. At our request, she discontinued the supplemental potassium three days before readmission for further study. At the end of this three-day period, serum potassium was 2.5 meq., blood pH 7.64, serum sodium 146 meq., chloride 101 meq., nonprotein nitrogen, 38, and serum creatinine 0.72 mg. per cent.

Fig. 2 demonstrates the persistent alkalosis which yielded only slightly during administration of potassium chloride as serum potassium rose to 3.3

meq. per liter. Note, too, that as serum potassium began to fall upon cessation of potassium supplements, serum sodium rose. There is some suggestion that ACTH precipitated a fall of serum potassium and a rise of serum sodium. The values at the right in Fig. 2 are those obtained after the twenty-nine-day period of potassium supplementation.

Fig. 3 shows the level of blood pressure, the variation being from 205/110 to 130/85. The major changes in hematocrit occurred during and after ACTH; hemodilution in the latter part of ACTH administration, followed by hemoconcentration on cessation of ACTH, followed again by spontaneous hemodilution.

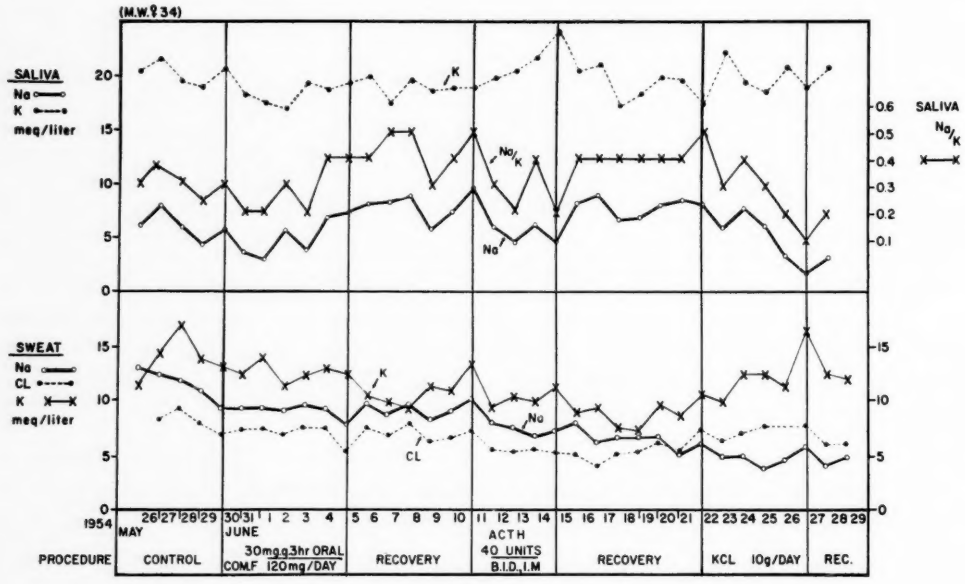



Fig. 1.—Electrolyte composition of daily samples of saliva and thermal sweat.

Fig. 4 shows that this patient's metabolic responses to compound F and to ACTH are perfectly normal (except for the electrolyte responses which will be shown in Figs. 5 and 6). Note first, that base-line values for 17-ketosteroids and for 17-hydroxysteroids are normal. Compound F, in the doses administered, produced, both times, a mild increase in urinary nitrogen, an increase in both 17-hydroxysteroids and 17-ketosteroids, a fall in eosinophils, and a rise in the fasting blood sugar. The post-compound F periods show positive nitrogen balance and return of all other values to normal.

Eighty units per day of ACTH produced, both times, greater and more persistent negative nitrogen balance and the expected large rises of urinary 17-ketosteroids and 17-hydroxycorticoid values. Upon cessation of ACTH, 17-hydroxycorticoids returned quickly to base line and 17-ketosteroids fell more slowly.

Fig. 5 shows data on renal excretion of electrolytes. In the control period there is a negative balance for potassium amounting to 12 meq. per day, and

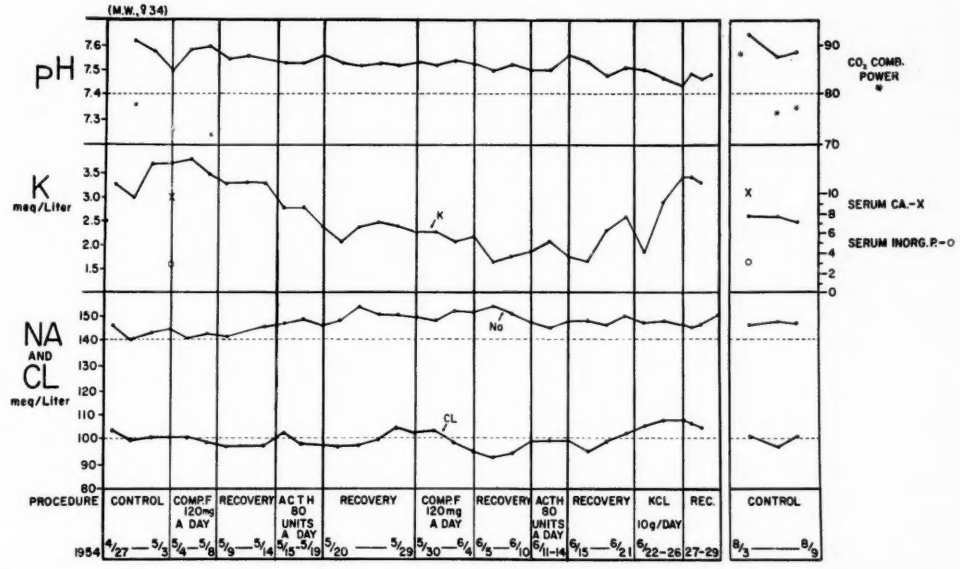



Fig. 2.—Blood pH, serum potassium, sodium, and chloride during 70 days of metabolic balance studies.

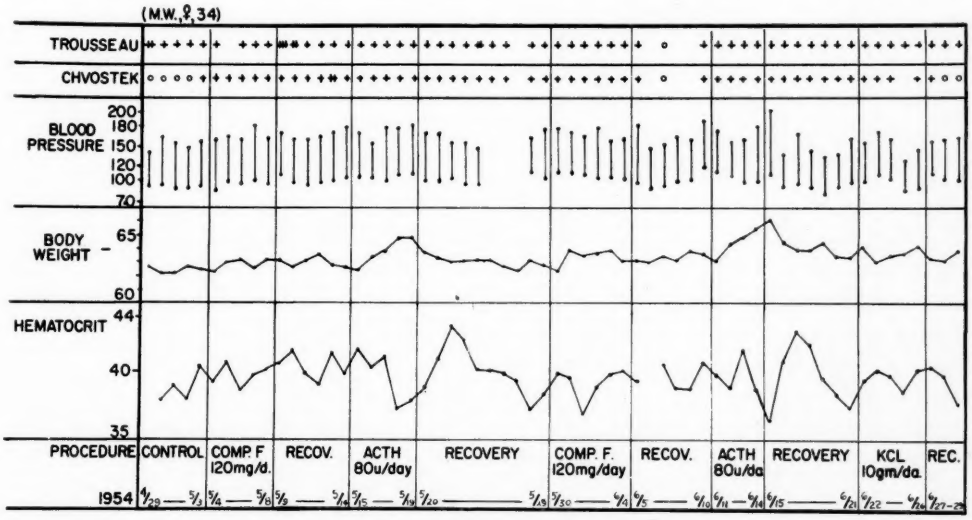



Fig. 3.—Blood pressure, body weight, and hematocrit.

there exists sodium equilibrium. Both compound F and ACTH increase potassium loss, but in each case the increased loss is either fully accounted for on the basis of the negative nitrogen balance (using a ratio of 2.7 meq. of potassium to 1 Gm. of extra nitrogen) or falls short of the expected amount. In normal people given these compounds, there occurs an initial loss of potassium in *excess* of nitrogen. This phenomenon is lacking in our patient, but it is to be recalled that she has been chronically in mild, negative potassium balance.

Of great interest in this patient is the effect of compound F and ACTH upon urinary excretion of sodium and chloride. After initial retention of sodium and chloride, there ensues a large outpouring of both of these ions while compound F or ACTH is still being administered. This is reminiscent of the hitherto unexplained phenomenon that some patients with nephrosis and edema (and presumably with high values for urinary sodium-retaining corticoid) demonstrate marked sodium and water diuresis while ACTH or cortisone is still being administered. In that instance, too, the diuretic phase is preceded by a period of sodium retention. *It would appear that in the presence of excessive activity of aldosterone, elevation of the 17-hydroxycorticoid level acts as an antagonist to aldosterone with respect to the direction in which the sodium ion moves across cell membranes.* Some years ago Thorn[15] talked of an apparent competition between desoxycorticosterone and cortisone in this respect, since he found that Addisonians on full doses of desoxycorticosterone lost sodium and chloride upon the addition of relatively large amounts of cortisone. Such amounts of cortisone administered without desoxycorticosterone produce sodium retention in Addison's disease.

One might argue in our case that compound F depressed endogenous ACTH and, thus, aldosterone, accounting for the sodium diuresis. There are two points against this. First, administration of ACTH gives the same response. Second, at the height of depression of the pituitary-adrenal axis in the post-ACTH or post-compound F periods, when 17-hydroxycorticoids are at their lowest levels, there is paradoxical sodium retention. Let me enlarge on this point.

The figures above the columns for urinary sodium represent the value for 17-hydroxycorticoid on that day. The normal post-ACTH or post-compound F response is sodium and chloride diuresis at the time of lowest adrenal cortical activity. Here, however, we observe paradoxical sodium retention at these times. This is highly suggestive that the sodium-retaining corticoid is still being secreted when endogenous ACTH is, at least, partially suppressed. Thus there appears to be some independence of secretion of this corticoid from the usual control. The fact that with ACTH, as opposed to compound F, higher levels of 17-hydroxycorticoids need to be produced to obtain sodium loss suggests that, in this patient, some increase in sodium-retaining corticoid is produced by exogenous ACTH; and that eventually 17-hydroxycorticoid production becomes sufficiently great in relation to the increase of sodium-retaining corticoid that loss of sodium occurs.

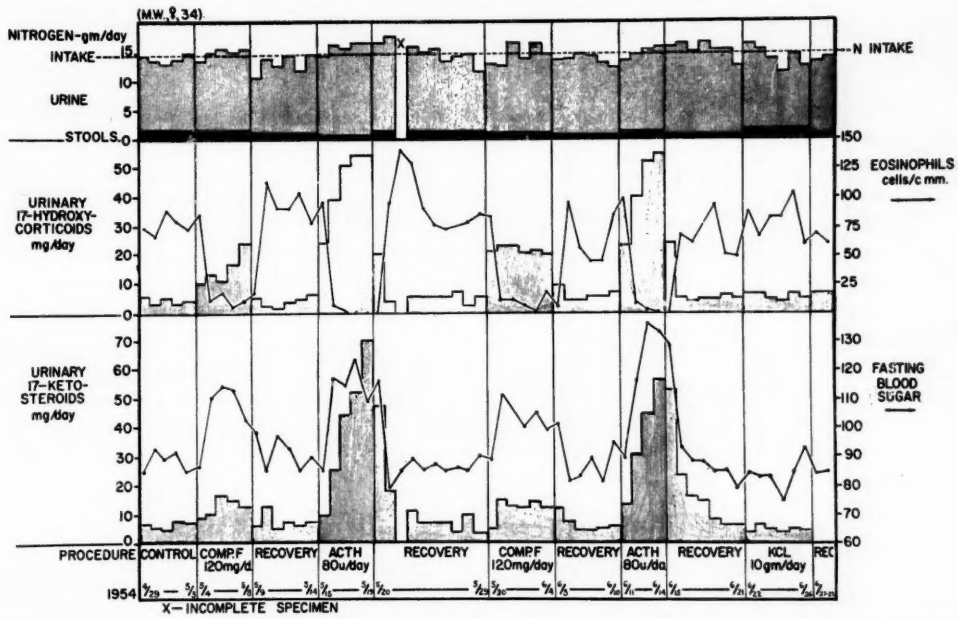



Fig. 4.—Nitrogen balance, 17-hydroxycorticoids, 17-ketosteroids, fasting blood sugar and eosinophils.

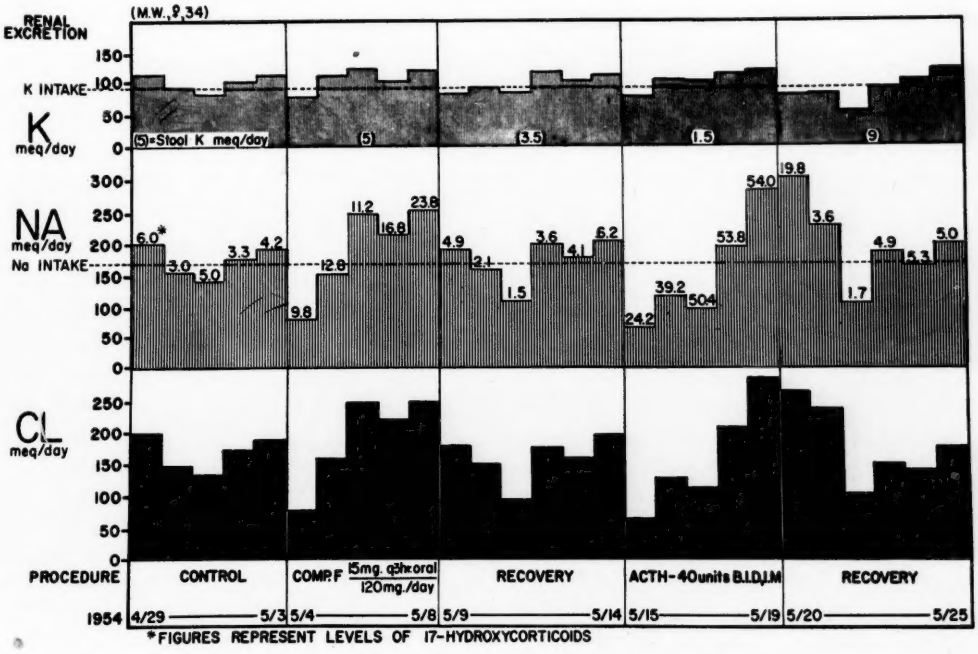



Fig. 5.—Renal excretion of sodium, potassium, and chloride.

Fig. 6 compares the immediate post-compound F periods of two normal men with those of our patient after compound F or ACTH. It is to be noted that in normal people sodium and chloride diuresis occurs when 17-hydroxycorticoid levels are lowest, while in M. W. the reverse is true.

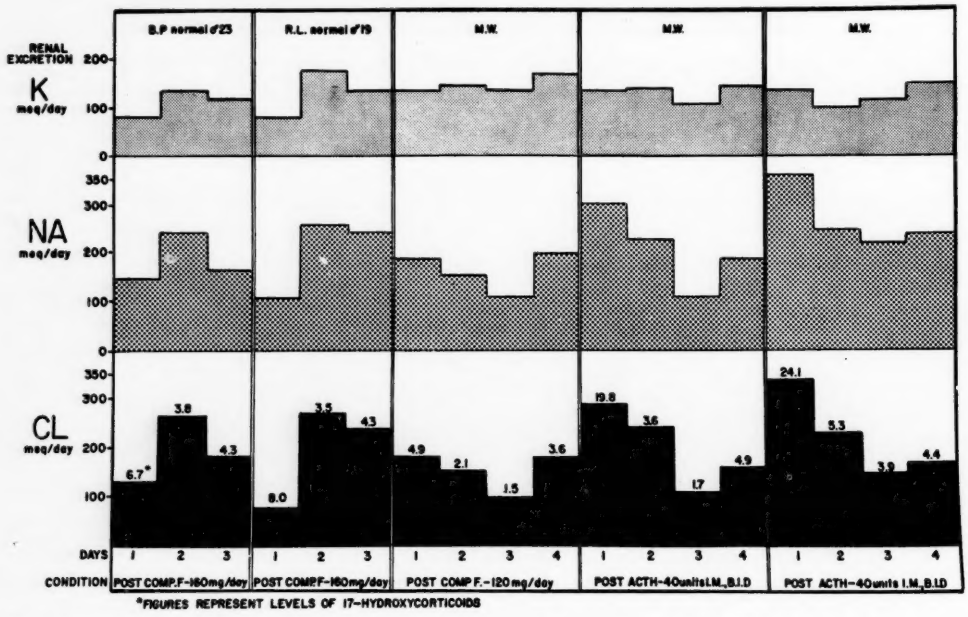



Fig. 6.—Comparison of urinary electrolyte patterns in normals and in M. W. upon cessation of compound F and ACTH.

Fig. 7 shows what happened when an attempt at potassium repletion was made. When a large amount of extra potassium was given, the initial response was the expected one, namely, retention of potassium and excretion of sodium. Actually, in the first four days 180 meq. of potassium were retained and 165 meq. of sodium were lost as the serum potassium level rose from 1.8 to 3.3 meq. per liter. At this point resistance to further sodium loss and further potassium retention occurred. In this period the salivary sodium-potassium ratio suggests a marked increase in activity of sodium-retaining corticoid. The values for sodium and chloride in sweat are already so low that they can hardly be expected to fall further. This combination of circumstances suggests that any attempt to alter the patient's newly established equilibrium, produced by chronic primary aldosteronism, meets quickly with resistance and with evidence of increased activity of sodium-retaining corticoid. This mechanism probably accounts for our inability to bring serum potassium values to normal with large amounts of supplementary potassium.

What we see in the recovery period, following administration of supplementary potassium, is what I picture as the metabolic aberration at the onset of this disease, namely, retention of sodium, loss of body potassium, a falling value for serum potassium, and a rising one for serum sodium. At some point

a new equilibrium is established with too little potassium and too much sodium in the body. The serum then exhibits the abnormalities typical of this syndrome. Attempts to alter this new equilibrium with potassium supplements meets with resistance because of the presence of excessive amounts of sodium-retaining corticoid.

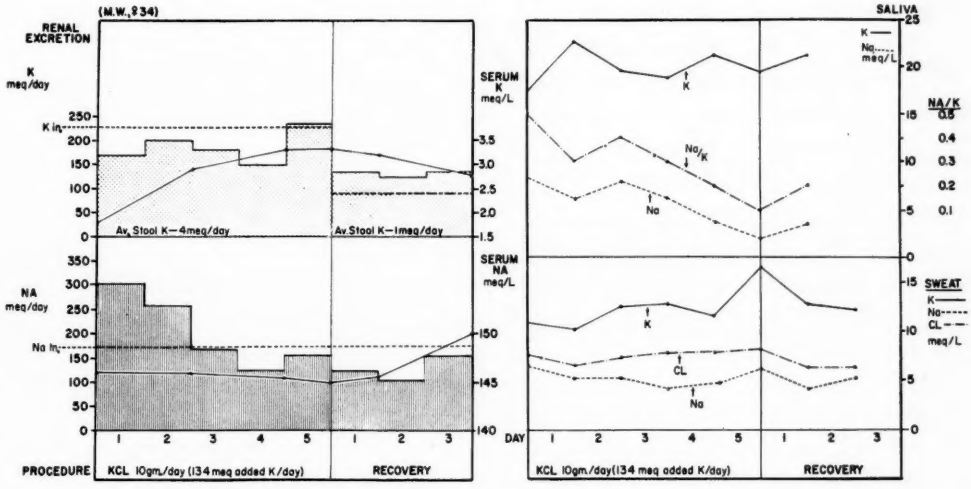



Fig. 7.—Effect of potassium chloride upon electrolyte balance and electrolyte composition of sweat and saliva.

It is not feasible, in the time that I have allotted for this presentation, to discuss the diverse clinical problems which lend themselves to further investigation on the basis of this concept of the metabolic activity of the sodium-retaining corticoid.

## SUMMARY

It is believed that these studies delineate a new clinical syndrome which is designated temporarily as primary aldosteronism. In its fully developed state it is characterized by the presence in the urine of excessive amounts of a sodium-retaining corticoid, by severe hypokalemia, hypernatremia, alkalosis, and a renal tubular defect in the reabsorption of water. The latter is probably secondary to chronic hypokalemia as reported by Schwartz and Relman.[13] The failure of renal concentrating ability is not corrected by pitressin.

When primary aldosteronism is fully established there exists only mild, negative balance for potassium in the presence of sodium equilibrium. Attempts to alter this newly established equilibrium by administration of potassium meets with resistance. With large doses of potassium, significant retention of this ion, for a few days, can be demonstrated along with a rising level of serum potassium and with excretion of sodium ion. This change toward normalcy, however, is quickly overcome by a shift in the opposite direction, namely, sodium retention and potassium loss. *Long-continued* administration

of potassium has no significant effect upon the abnormal serum values or upon the symptomatology of the syndrome. It seems likely that resistance to potassium repletion is due to an inability of cells to release an excessive amount of intracellular sodium which is being held there by excessive activity of sodium-retaining corticoid. In the presence of excessive activity of aldosterone, high 17-hydrocorticoid levels, induced by either compound F or ACTH, produce sodium diuresis.

The clinical picture consists of intermittent tetany, paresthesia, periodic severe muscular weakness and "paralyses," polyuria and polydipsia, hypertension, and no edema. Of additional interest is the *relative* lack of important symptomatology at extremely low levels of serum potassium. The syndrome can occur in the absence of any other demonstrable evidence of increased activity of the adrenal cortices.

Small amounts of urinary protein may appear. This, too, may be related to the renal tubular defect, secondary to chronic hypokalemia or it may be an independent effect of excessive amounts of sodium-retaining corticoid. It is suggested that the interesting cases reported by Earle and associates[3] and by Wyngaarden and associates[4] as so-called "potassium-loss nephritis" represent a later stage of primary aldosteronism.

From a therapeutic point of view and in the light of present knowledge, these data indicate that total adrenalectomy followed by substitution therapy should abolish the entire metabolic abnormality. The patient is now in the hospital in preparation for this procedure. We hope to report other studies, as well as the results of adrenalectomy, at a later date.

## ADDENDUM

Subsequent to submission of this paper for publication there has appeared from England a careful study by B. M. Evans and M. D. Milne entitled, "Potassium-Losing Nephritis Presenting as a Case of Periodic Paralysis" (Brit. Med. J. 2: 1,067, 1954). The data contained therein indicate that the patient studied has the same abnormalities which we have described above as being associated with *Primary Aldosteronism.* Of exceedingly great interest is the fact that Evans and Milne state "Bioassay of electrocortin excretion gave abnormally high figures." But no further comment in this regard is made. Plasma levels of 17-hydroxycorticoids and urine values for Compounds E and F were found to be normal.

## ADDITIONAL ADDENDUM

On Dec. 10, 1954, operation was performed on M. W. A right adrenal cortical tumor, 4 cm. in diameter was removed. The left adrenal gland was left in situ. Further studies are now in progress.

## REFERENCES

1. Bean, W. B.: A Testment of Duty, J. Lab. & Clin. Med. **39**: 3, 1952.
2. Mader, I. J., and Iseri, L. T.: Spontaneous Hypopotassemia, Hypomagnesemia, Alkalosis and Tetany (Abstract 112), Proc. Central Soc. Clin. Research **27**: 81, 1954.
3. Earle, D. P., Sherry, S., Eichna, L. W., and Conan, N. J.: Low Potassium Syndrome Due to Defective Renal Tubular Mechanisms or Handling Potassium, Am. J. Med. **11**: 283, 1951.
4. Wyngaarden, J. B., Keitel, H. G., and Isselbacher, J.: Potassium Depletion and Alkalosis. Their Association With Hypertension and Renal Insufficiency, New England J. Med. **250**: 597, 1954.

5. Conn, J. W.: The Mechanism of Acclimatization to Heat. Advances in Internal Medicine, New York, 1949, Interscience Publishers Inc., Vol. 3, pp. 373-393.
6. Conn, J. W., and Louis, L. H.: Production of Endogenous Salt-Active Corticoids as Reflected in the Concentrations of Sodium and Chloride of Thermal Sweat, J. Clin. Endocrinol. **10:** 12, 1950.
7. Simpson, S. A., Tait, J. F., and Bush, I. E.: Lancet **263:** 226, 1952.
8. Deming, Q. B., and Luetscher, J. A., Jr.: Bioassay of Desoxycorticosterone-like Material in Urine, Proc. Soc. Exper. Biol. & Med. **73:** 171, 1950.
9. Chart, J. J., and Shipley, E. G.: The Mechanism of Sodium Retention in Cirrhosis of the Liver, J. Clin. Invest. **32:** 560, 1953.
10. Singer, B., and Venning, E. H.: Method of Assay of a Sodium-Retaining Factor in Human Urine, Endocrinology **52:** 623, 1953.
11. Luetscher, J. A., Jr., and Johnson, B. B.: Chromatographic Separation of the Sodium-Retaining Corticoid From the Urine of Children With Nephrosis, Compared With Observations on Normal Children, J. Clin. Invest. **33:** 276, 1954.
12. Silber, R. H., and Porter, C. C.: The Determination of 17, 21-Dihydroxy-20-Ketosteroids in Urine and Plasma, J. Biol. Chem. **210:** 923, 1954.
13. Schwartz, W. B., and Relman, A. S.: Metabolic and Renal Studies in Chronic Potassium Depletion Resulting From Overuse of Laxatives, J. Clin. Invest. **32:** 258, 1953.
14. Luetscher, J. A., Jr., and Axelrad, B. J.: Sodium-Retaining Corticoid in the Urine of Normal Children and Adults and of Patients With Hypoadrenalism or Hypopituitarism, J. Clin. Endocrinol. **14:** 1086, 1954.
15. Thorn, G. W., Forsham, P. H., Bennett, L. L., Roche, M., Reiss, R. S., Slessor, A., and Flink, E. B.: Clinical and Metabolic Changes in Addison's Disease Following the Administration of Compound E Acetate, Proc. A. Am. Physicians **62:** 235, 1949.

# CLINICAL AND EXPERIMENTAL

## THROMBOCYTOPENIC PURPURA CAUSED BY HYPERSENSITIVITY TO QUININE

RUTH STEINKAMP, M.D.,* CARL V. MOORE, M.D., AND
WILLIAM G. DOUBEK, M.D.,**
ST. LOUIS, MO.

INTEREST in the pathogenesis of drug-induced thrombocytopenic purpura has been stimulated by the studies of Ackroyd.[1-3] He demonstrated that when thrombocytopenia was produced by sensitivity to Sedormid, the in vitro addition of Sedormid to blood during the recovery period caused agglutination of platelets. In the presence of complement, lysis of platelets occurred. Both reactions were dependent on a plasma factor, presumably an antibody, in the blood of the sensitized patient. These observations have recently been extended to quinidine-induced purpura.[4, 5] The present report describes similar results obtained on a patient who developed thrombocytopenia because of sensitivity to quinine. In addition to in vitro demonstration of platelet agglutination and the experimental production of purpura by a test dose of quinine, thrombocytopenia was induced in a normal subject by giving him quinine by mouth and a transfusion of the patient's plasma.

Other instances of thrombocytopenic purpura due to quinine have been reported.[6-15] Quinine sensitivity was proved in several of these cases by giving a test dose to reproduce the purpura,[7, 13, 14] or by demonstrating platelet agglutinins or lysins in vitro.[13, 15] Peshkin and Miller[8] in 1934 first suggested that the thrombocytopenia caused by quinine was a drug allergy because they were able to produce positive skin and passive transfer tests. Subsequent investigators have obtained either positive passive transfer tests in similar cases,[9] or positive intradermal tests.[12] Note should also be made of the fact that Rappoport, Nixon, and Barker[16] suggested in 1945 that purpura occurring in a patient after sodium salicylate administration might have an antigenantibody mechanism.

### CASE REPORT

D. F., a white woman 31 years of age, was admitted to St. Louis County Hospital for the first time on May 5, 1953, because of uncontrolled expistaxis of six hours' duration. She had noted small red spots on her lower extremities one week before at the time her normal menses began. The latter lasted two days but the red spots persisted. She had taken large quantities of a proprietary analgesic (Bromoseltzer†), for five to six years,

From the Department of Medicine, Washington University, St. Louis, Mo.

This study was supported in part by Research Grant H22 from the National Heart Institute of the National Institutes of Health, Public Health Service.

Received for publication July 2, 1954.

*National Cancer Institute Trainee.

**St. Louis County Hospital.

†Bromoseltzer, marketed by Emerson Drug Co., Baltimore, Md., contains the following per heaping teaspoonful: Acetanilid 2½ grains, sodium bromide 5 grains, caffeine, sodium bicarbonate, and citric acid.

and an occasional tablet of Bromoquinine,* usually at onset of her menstrual period. During the eighteen to twenty hours immediately prior to admission, she had taken twelve tablets of Bromoquinine.

The past history and family history were completely negative for any blood diseases, bleeding tendencies, or allergic manifestations. The system review was negative except for frequent headaches associated with eyestrain, for which she took the Bromoseltzer.

Physical examination revealed a blood pressure of 104/85 mm. Hg, pulse 82 per minute, respirations 22 per minute. Temperature was slightly elevated. She was an obese white woman with numerous petechiae over the lower extremities. There was profuse bleeding from both nostrils, the gingivae, and the uterus. While in the emergency room, she lost approximately 250 ml. of blood by epistaxis and vomited 500 ml. of bloody material.

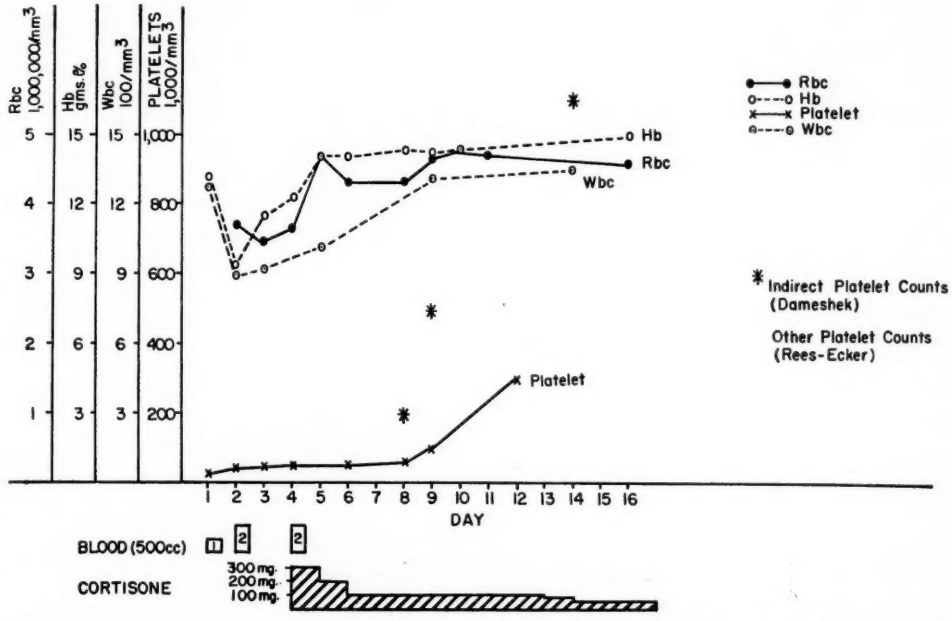



Fig. 1.—Peripheral blood elements and course of therapy in a quinine-sensitive patient following the first acute episode of thrombocytopenic purpura.

The hemogram showed 13.2 Gm. of hemoglobin per 100 ml., 12,850 leukocytes per cubic millimeter, and 32,000 platelets per cubic millimeter (Rees-Ecker method), and a differential count of band forms 17 per cent, polymorphonuclear neutrophils 70 per cent, lymphocytes 12 per cent, monocytes 1 per cent. The bleeding time was six minutes and coagulation time (Lee-White method) four minutes. The urine was clear.

Immediate treatment consisted of intravenous 5 per cent glucose in 0.9 per cent saline, blood transfusion of 1,000 ml., and nasal packs. During the first twenty-four hours of hospitalization another hematemesis of about 500 ml. occurred; the hemoglobin dropped to 9.4 Gm. per 100 ml., and an additional 1,000 ml. of blood was given. The platelet count twenty-four hours after admission was 46,000 per cubic millimeter. The prothrombin time was 22 seconds. Coombs' test was negative.

On the second hospital day a tarry stool was passed, and she was given another 1,000 ml. of blood. Fig. 1 summarizes the peripheral blood counts and the treatment. Cortisone

*Bromoquinine tablets, marketed by Grove Laboratories, Inc., St. Louis, Mo., contain the following per tablet: Acetphenetidin 1¾ grains, extract belladonna 1/50 grain (1.25 per cent total alkaloids), extract hyoscyamus 1/16 grain (0.155 per cent total alkaloids), quinine hydrobromide ⅜ grain caffeine, yellow phenolphthalein, and oleoresin capsicum.

therapy was started on the third hospital day. By the fifth hospital day bleeding had stopped. Bone marrow examination at that time revealed a cellular marrow with morphologically normal but moderately hyperplastic myeloid and erythroid elements. Many megakaryocytes were seen at all maturation levels. There was a slight increase in plasma cells, but no significant increase in eosinophilic elements. The platelet count was 108,000 per cubic millimeter by the ninth day.

During the next five months the patient had no evidence of abnormal bleeding and remained completely well except for recurrent headaches. She had avoided all medications. However, on Oct. 18, 1953 (on the second day of her normal menstrual period), she developed a severe headache and slight abdominal cramping. She then took a large spoonful of Bromoseltzer and one tablet of Bromoquinine. About twenty hours later she began to bleed profusely from the nose and gums; the menstrual bleeding became more profuse and she developed petechiae on her legs and chest. Her private physician advised her to return to the St. Louis County Hospital. Soon after admission she vomited approximately 600 ml. of dark blood. Physical examination revealed an acutely ill, normotensive woman with a temperature of 39° C. There were petechiae on the hard palate, anterior chest, thighs, lower legs, and feet. Acneiform eruptions on the face were dark red and encrusted. There was profuse bleeding from the gums, nose, and vagina. The remainder of the examination was negative.

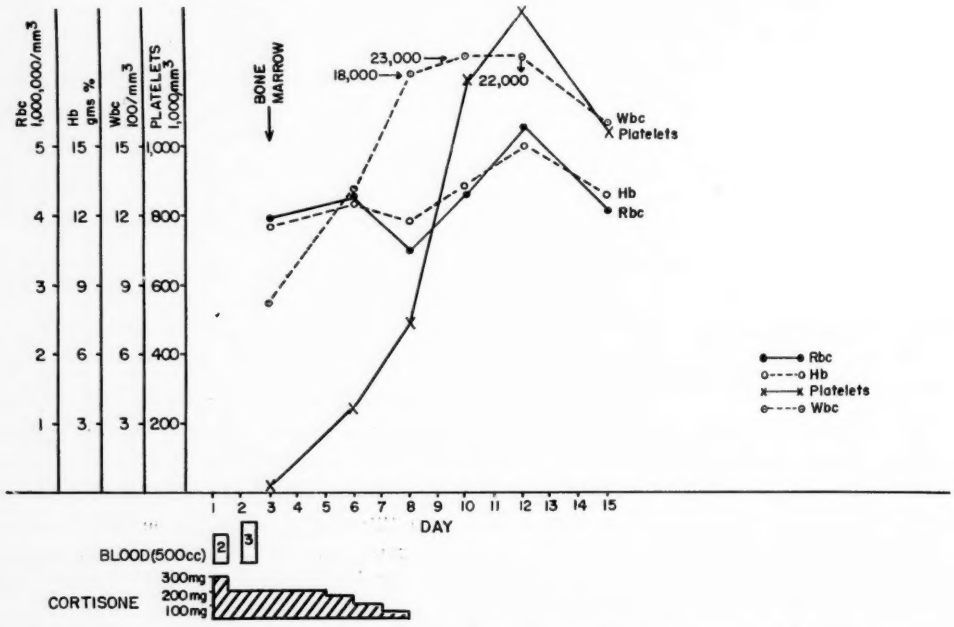



Fig. 2.—Peripheral blood elements and course of therapy in a quinine-sensitive patient following the second acute episode of thrombocytopenic purpura.

The hemoglobin on admission was 12.1 Gm. per 100 ml. and only a few platelets were seen on a stained blood film. The urine was negative. Fig. 2 presents the course of the peripheral blood elements and her therapy. Bone marrow examination three days after admission was similar to that of the previous admission. The platelet count on the third day was 20,000 per cubic millimeter, but by the sixth day it had risen to 258,000 (Dameshek method).

On the first admission the question arose that this was symptomatic and not idiopathic purpura because: (1) drugs known to cause purpura had been ingested, (2) the

platelet count rose rapidly, and (3) the patient's serum caused in vitro agglutination (vide infra) of only one-third of the number of platelet suspensions against which it was tested.

Platelet counts were done by the Dameshek method[17] of indirect counting on the patient's second admission and in the studies of the normal subject. During the patient's first admission, platelets were counted by the Rees-Ecker[18] method of direct counting.

In vitro platelet agglutinin tests were performed, using the method of Harrington and associates.[19]

Bromoseltzer compound, pure acetanilid, sodium bromide, and quinine hydrobromide for the in vitro platelet agglutinin tests were used in concentrations which would have been reached in the extracellular fluid if it is assumed that the drug was completely absorbed and the total amount distributed in the patient's calculated extracellular fluid volume. The following amounts were dissolved in 100 ml. physiologic saline solution: acetanilid, 40 mg.; sodium bromide, 80 mg.; Bromoseltzer, 1 Gm.; quinine hydrobromide, 6 mg. and 20 mg., respectively. One-tenth milliliter of each solution was used in the in vitro platelet agglutinin test. At these concentrations, the pH was well within the range recommended for the method.

## MATERIALS AND METHODS

The patient's plasma for in vivo tests was maintained at 5° C. for two and five days, respectively, before the tests were performed. That utilized for the in vitro platelet agglutinin tests was frozen and maintained at -10° C. Just before each test, the plasma was thawed at a low temperature and was later refrozen for further in vitro tests.

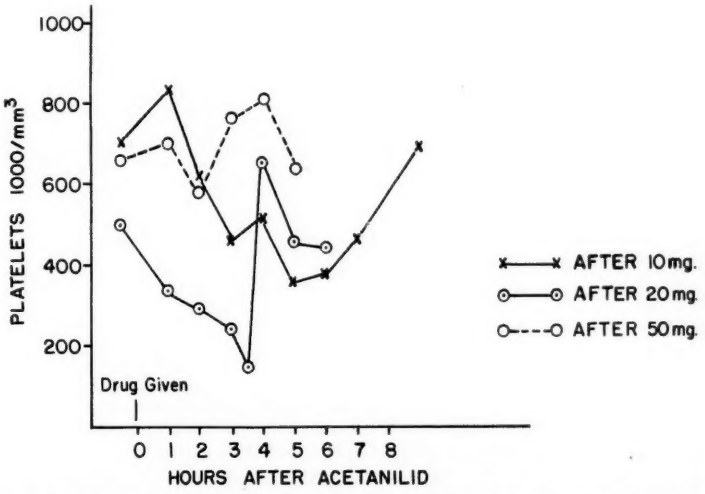



Fig. 3.—Response of platelets in a quinine-sensitive individual to oral test doses of 10 mg., 20 mg., and 50 mg. acetanilid.

## RESULTS

I. *In Vivo Studies To Determine the Sensitizing Drug in the Patient.*—Since the drug history on the second admission was at first limited to Bromoseltzer, an attempt was made to incriminate one of its constituents as the offending agent.

A. *Results of oral test doses of acetanilid* (Fig. 3): On three different occasions, acetanilid was given orally at varying dosages. Following a 10 mg. dose, the platelets dropped approximately 50 per cent in five hours; a 20 mg. dose caused a 70 per cent reduction in three and one-half hours. On neither occasion was this a reduction to lower than the minimum normal levels. A

third dose of 50 mg. caused no essential change in the platelet count. Bone marrow examinations after the first two doses of acetanilid showed no abnormality of the megakaryocyte.

It was during the course of these tests that the patient admitted having taken one tablet of Bromoquinine just prior to the last episode of thrombocytopenic purpura. Among the several drugs contained in this compound is quinine hydrobromide.

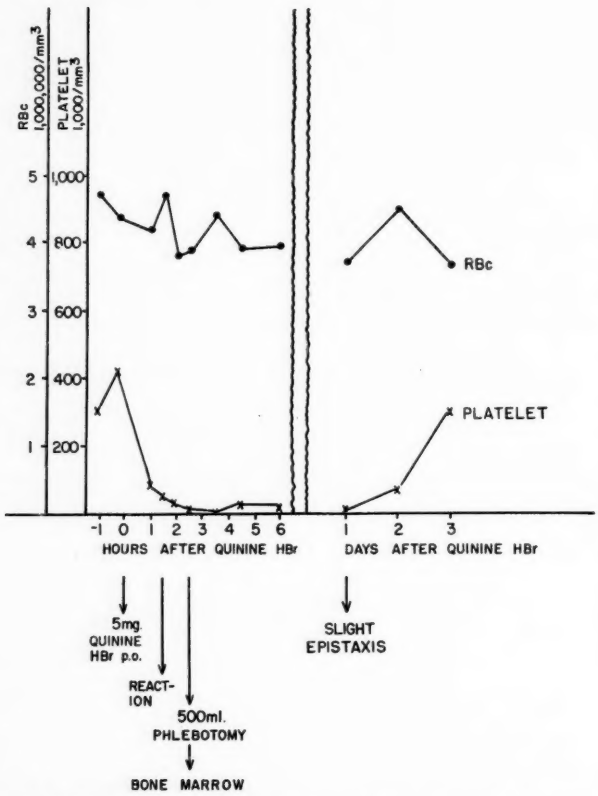



Fig. 4.—Response of platelets and erythrocyte counts in a quinine-sensitive individual to an oral test dose of quinine hydrobromide (5 mg.).

B. *Result of an oral test dose of quinine hydrobromide* (Fig. 4): Five milligrams of quinine hydrobromide (one-fifth of the amount in one tablet of Bromoquinine) were given by mouth. One and one-half hours after taking the drug, the patient had a shaking chill, developed headache and generalized abdominal aching, became nauseated, vomited once, and had a temperature rise to 100.1° F. The platelet count prior to the dose was 400,000 per cubic millimeter. After one hour it was 145,000 and after three and one-half hours it reached its lowest level, 17,600. The bleeding time was 12 minutes. During the first two days after this acute episode, the platelets were more granular than normal. As they rose in number, a few giant forms were seen. The count was normal on the third day. Bone marrow examination during the time of the marked reduction of platelets showed little change in the meg-

akaryocytes from normal. There was a slight increase in number and the nuclei of a few megakaryocytes appeared moderately immature. At the time of the lowest platelet count, a 500 ml. phlebotomy was performed. The plasma was transfused later into a normal individual.

II. *Platelet Responses of a Normal Individual Following Simultaneous Administration of Oral Quinine Hydrobromide and Intravenous Injection of Plasma From a Quinine-Sensitive Patient and Control Studies on the Same Subject With Administration of These Materials.*—A normal volunteer orally ingested 500 mg. quinine hydrobromide three hours before 200 ml. of the patient's plasma was administered intravenously (Fig. 5). An additional 250 mg. of the

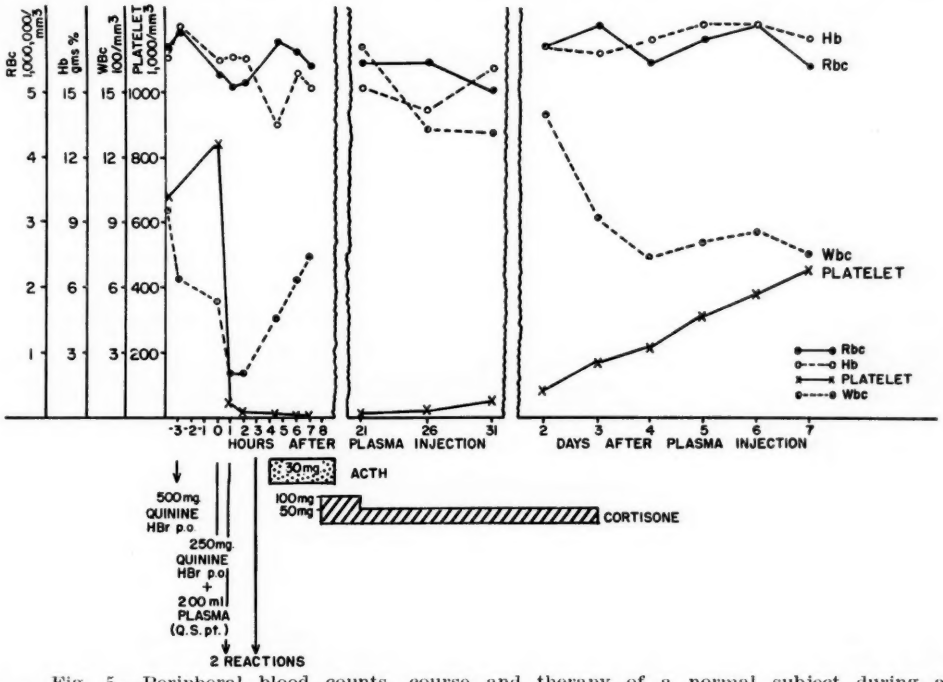



Fig. 5.—Peripheral blood counts, course and therapy of a normal subject during a test dose (5 mg.) of quinine hydrobromide following test with simultaneous administration of quinine and quinine-sensitized plasma.

drug was taken as the infusion of plasma was begun. One hour later, the subject felt chilly and developed a headache. Five minutes after this, a severe reaction occurred consisting of a shaking chill, increase in headache, severe lumbar and sacral pain, and cyanosis. The reaction lasted ten minutes. The temperature rose from normal to 38.5° C. Two hours later a similar reaction occurred and continued for fifteen minutes. In addition, nausea and vomiting developed. Because of the marked drop in the platelet count from 850,000 to 20,000 per cubic millimeter, oral cortisone and an intravenous infusion of ACTH in saline were given. During the next twenty-four hours, the subject continued to have fever to 39° C. Generalized petechiae developed and the gums bled slightly. The bleeding time was greater than 18 minutes. The platelet count was 5,400 at seven hours, 16,000 at twenty-one hours, and 56,000 at thirty-one hours after the plasma had been given. During the time of

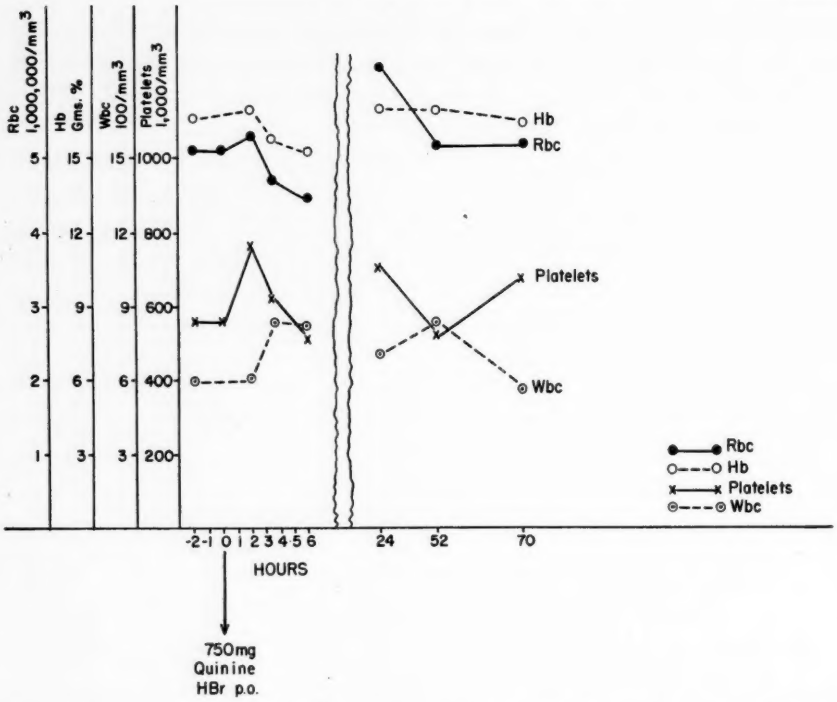



Fig. 6.—Peripheral blood counts of a normal subject receiving quinine hydrobromide alone (750 mg.).

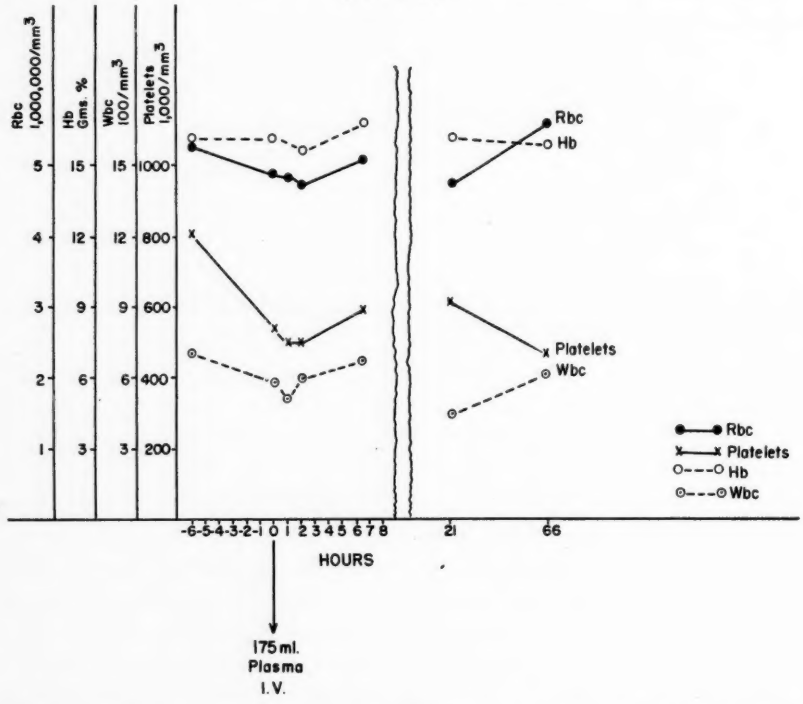



Fig. 7.—Peripheral blood counts of a normal subject receiving plasma alone from a quinine-sensitized donor.

the lowest counts, the few platelets seen were ragged and had a granular cytoplasm. The platelets increased steadily in number and were completely normal in quantity and morphology seven days after the test had been performed.

For control studies, three months after the original test, the same volunteer was given quinine hydrobromide orally and on another occasion 175 ml. of plasma from the recovered quinine-sensitive patient. Figs. 6 and 7 show the platelet and red cell responses to these test substances. Following the oral intake of 750 mg. quinine hydrobromide, there was essentially no change in the platelet count during the subsequent six-hour period or at intervals up to seventy hours after the dose. Similar results were obtained after administration of the plasma alone. The latter would have been a better control if this plasma had also been obtained during a thrombocytopenic phase rather than after the patient had recovered. However, addition of quinine in vitro caused marked agglutination of platelets so the antibody was still present (vide infra).

III. *In Vitro Platelet Agglutinin Tests.*—Tests for platelet agglutinins were performed according to the method of Harrington and associates[19] on samples of sera obtained from the patient at varying intervals after the manifestations of acute thrombocytopenic purpura. Platelet-rich sera were obtained from normal individuals for agglutinin tests, 10 ml. of blood being collected in 0.3 ml. of 5 per cent "Sequestrene"* anticoagulant. Reading of the test is based on the degree of clumping of platelets from "negative" to a "4-plus" reaction. Complement is required for lysis of platelets as shown by Ackroyd.[3] "Sequestrene" is anticomplementary in the dilution used; therefore no lysis of platelets was to be expected.

Table I presents the results of platelet agglutinin tests performed on sera of the quinine-sensitive patient at varying times, and when quinine was added to the system. Tests on serum obtained three days after the first thrombocytopenic episode showed that of fifteen platelet suspensions tested, eleven were negative and four were positive to a 1-plus to 2-plus reaction. The sera obtained following the second episode of thrombocytopenic purpura gave similar results. However, that drawn at the time of the acute reaction to a test dose of quinine was positive in four of five platelet suspensions to a 2-plus to 3-plus reaction. The difference may have been due to a transient presence of quinine in the blood, since after recovery from this episode only one platelet suspension of the twenty-two tested was positive.

Additions to the patient's plasma and platelet suspensions of the various components of the known ingested drugs caused significant and consistent agglutination only in those to which quinine was added. In all these there was a 4-plus agglutination. On the other hand, with the Bromoseltzer and its consistuents, acetanilid and sodium bromide, no consistent agglutination was obtained. Quinidine, the optical isomer of quinine caused no agglutination of platelets when added to the patient's serum.

*Sequestrene, $Na_2$, is the trade name for ethylene diaminetetraacetic acid, disodium salt; Alrose Chemical Company, Providence, R. I.

TABLE I. IN VITRO PLATELET AGGLUTININS IN SERUM OF QUININE-SENSITIVE PATIENT

| TIME AT WHICH SERUM WAS OBTAINED | TYPE OF TEST | TOTAL NO. PLATELET SUSPENSIONS TESTED | NO. SUSPENSIONS AGGLUTINATED | NO. SUSPENSIONS NOT AGGLUTINATED |
|---|---|---|---|---|
| Three days after first episode of acute T.P. | Serum alone | 15 | 4 | 11 |
| Two days after second episode of acute T.P. | Serum alone | 7 | 2 | 5 |
| Seven and 4 days after 10 mg. and 20 mg. acetanilid P.O. | Serum alone | 10 | 1 | 9 |
| During acute reaction to test dose of quinine HBr | Serum alone | 5 | 4 | 1 |
| Complete recovery from second attack of T.P. and reaction to quinine test dose. | Serum alone | 22 | 1 | 21 |
| During acute reaction to test dose of quinine HBr | Serum + quinine | 6 | 6 | 0 |
| Complete recovery from second attack of T.P. and from reaction due to test dose of quinine HBr | Serum + quinine (6 mg. level) | 4 | 4 | 0 |
| Complete recovery from second attack of T.P. and from reaction due to test dose of quinine HBr | Serum + quinine (20 mg. level) | 4 | 4 | 0 |

T. P. = Thrombocytopenic purpura.

Bigelow and Desforges[20] demonstrated in vitro agglutination of platelets in two cases of quinidine purpura by similar tests only when the offending drug was added to the system. In one of their cases the factor was not present six months later and in the other it was absent after thirty-nine days. In the latter case, the factor survived storage at –20°C. for forty days. Barkham and Tocantins[5] found that in their case of quinidine purpura in vitro hypersensitivity was persistent for four weeks but was lacking at six weeks. Similar studies in our patient revealed the factor to be present in a high panagglutinin titer in serum obtained 203 days after recovery from the drug and to have withstood storage at –10° C. for 199 days.

Similar tests on normal sera with and without added drugs are presented in Table II. The few positive tests obtained were of a 1-plus reaction.

TABLE II. IN VITRO PLATELET AGGLUTININS IN SERUM OF NORMAL SUBJECT AND IN SUBJECT RECEIVING QUININE HBR AND PLASMA FROM QUININE-SENSITIVE PATIENT

| TIME INTERVAL | SERUM TESTED | TOTAL | PLATELET AGGLUTINATION | |
|---|---|---|---|---|
| | | | POSITIVE* | NEGATIVE |
| | N.S.† | 5 | 0 | 5 |
| | N.S. + Bromoseltzer | 4 | 1 | 3 |
| | N.S. + NaBr | 4 | 0 | 4 |
| | N.S. + acetanilid | 4 | 0 | 4 |
| | N.S. + quinine HBr (6 mg.) | 5 | 1 | 4 |
| | N.S. + quinine HBr (20 mg.) | 4 | 0 | 4 |
| 16 hours after reaction | N.S. | 5 | 0 | 5 |
| 16 hours after reaction | N.S. + quinine HBr (20 mg.) | 5 | 1 | 4 |
| 5 days after reaction | N.S. | 5 | 2 | 3 |
| 5 days after reaction | N.S. + quinine HBr (20 mg.) | 5 | 1 | 4 |

*Positive agglutinations were either 1-plus or plus-minus reactions.
†Normal serum.

IV. *Results of Passive Transfer Tests.*—Passive transfer tests were attempted on two different occasions on the same normal individual. In the first test, 0.1 ml. of the patient's serum, obtained immediately after the reaction to the test dose of quinine hydrobromide, was injected intracutaneously into each of three different sites. Forty-eight hours later, 0.1 ml. of a 1 per cent solution of quinine hydrobromide was injected into the same sites. For the second test, three different intracutaneous sites were injected with the same serum used in the first test. Forty-eight hours later, the recipient's serum incubated for two hours with the drug was injected into the same sites. Control injections with quinine hydrobromide in saline and quinine hydrobromide in recipient's serum were also given. In none of the injected areas was a positive test obtained.

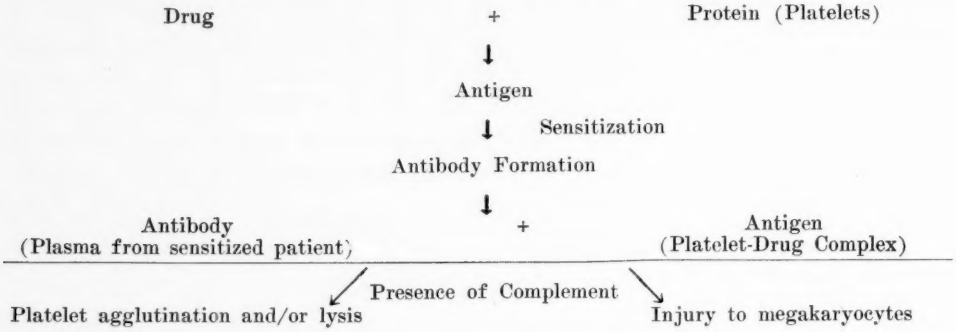
## DISCUSSION

Thrombocytopenic purpura developing in an individual on two occasions after repeated ingestion of Bromoseltzer and Bromoquinine over a long period of time has been shown to be due to a specific hypersensitivity to quinine. When a test dose of the drug was given to the patient, and when both quinine and the patient's plasma were administered to a normal recipient, the platelet counts fell to low levels within an hour. Since platelets normally have an in vivo survival time of about five days, destruction of platelets by the immune mechanism must have occurred.

In addition, since the thrombocytopenia persisted for several days, bone marrow megakaryocytes must also have been damaged. Such an effect would be expected since an antibody which injures platelets would probably also react with the cytoplasm of platelet-producing cells. The change would be similar to the megakaryocytic damage observed by Sprague and associates,[21] when plasma from patients with idiopathic thrombocytopenic purpura was transfused into normal recipients. Hirsch and Dameshek,[22] furthermore, have described morphologic evidence of injury to megakaryocytes in a patient with quinidine-induced thrombocytopenia. Unfortunately, the bone marrow in our patient was not examined until five and three days, respectively, after the onset of the two episodes of thrombocytopenia, and we probably missed the more profound changes. Even so, young megakaryocytes—probably indicative of recovery after the initial damage—were observed. No bone marrow studies were made on the normal recipient after the quinine-plasma induced thrombocytopenia.

Attempts by Moeschlin[23] to induce thrombocytopenia in normal subjects by transfusing them with blood from patients who had developed purpura because of Sedormid hypersensitivity were unsuccessful. The evidence presented here suggests that if the normal recipients had been given Sedormid at the time of the transfusion, they too might have become thrombocytopenic. Larson has suggested that the responsible drug serves as a haptene to induce a state of sensitivity with the production of antibodies.[4] One may postulate, as

Moeschlin and associates[24] have done for immunoagranulocytosis, that the mechanism of thrombocytopenia caused by drug sensitivity is as follows:




The platelet antibody, in this type of reaction, differs from the platelet autoagglutinins and isoagglutinins described by Harrington and associates[19] in that a third substance—the sensitizing drug—is an essential component of the antigenantibody reaction.

In vitro platelet agglutinin tests were found to be consistently positive with plasma from the quinine-sensitive individual only when quinine was added to the test substances. Quinidine, the optical isomer of quinine, was not effective. This highly specific reaction was noted also by Larson[4] and Barkham and Tocantins[5] in that quinine did not cause platelet agglutination when added to the sera of quinidine-sensitive patients.

Intriguing is the possibility of disappearance of the hypersensitive state and its correlation with disappearance of the demonstrable platelet agglutinins. Previous workers[5, 20] have demonstrated loss of platelet agglutinins but have been unable to repeat in vivo tests to note patient's response. In our case, the platelet agglutinins were still demonstrable after 203 days.

## SUMMARY

Studies are reported on a patient who developed thrombocytopenic purpura on two separate occasions after ingestion of Bromoseltzer and Bromoquinine, the latter containing quinine hydrobromide. After recovery from the second acute episode, test doses with acetanilid, a constituent of Bromoseltzer, failed to produce a significant platelet reduction, whereas 5 mg. of quinine hydrobromide caused a systemic reaction, a marked reduction in platelets, and prolongation of bleeding time.

Marked thrombocytopenic purpura with a prolonged bleeding time and petechiae was produced in a normal volunteer after concomitant oral ingestion of quinine hydrobromide and intravenous injection of plasma from the quinine-sensitive patient. No change in platelet counts was observed in the same volunteer when quinine or plasma from the patient was given alone. Platelet agglutinin tests done on plasma from the recipient were negative.

In vitro platelet agglutinin tests were consistently positive only when quinine hydrobromide was added to the patient's serum. The serum factor remained active after 199 days' storage at –10° C. In the presence of quinine, a high panagglutinin titer could still be demonstrated in the patient's blood

drawn 203 days after recovery from the second acute episode of thrombocytopenic purpura. No conclusive platelet agglutinins were demonstrable when sodium bromide, acetanilid, or Bromoseltzer were added to the patient's serum.

Passive transfer tests using patient's serum and quinine were negative.

It is suggested: (a) that the immune mechanism responsible for the thrombocytopenia causes both a peripheral destruction of platelets and transient damage to megakaryocytes, and (b) that the antigen to which antibody is produced is a complex composed of a union between the drug and platelets.

Grateful acknowledgment is given to Miss Virginia Minnich and Dr. Supa Na-Nakorn for conducting the in vitro platelet agglutinin tests and to Miss Mary Johnson for performing the passive transfer tests.

REFERENCES

1. Ackroyd, J. F.: The Cause of Thrombocytopenia in Sedormid Purpura, Clin. Sc. **8**: 269-290, 1949.
2. Ackroyd, J. F.: The Pathogenesis of Thrombocytopenic Purpura Due to Hypersensitivity to Sedormid (Allyl-isopropyl-acetyl-carbamide), Clin. Sc. **7**: 249-285, 1949.
3. Ackroyd, J. F.: The Role of Complement in Sedormid Purpura, Clin. Sc. **10**: 185-208, 1951.
4. Larson, R. K.: The Mechanism of Quinidine Purpura, Blood **8**: 16-25, 1953.
5. Barkham, P., and Tocantins, L. M.: Observations on the Thrombocytopenia Due to Hypersensitivity to Quinidine, Blood **9**: 134-143, 1954.
6. Regendanz, P.: Ueber Erkrankungen nach Chinin. Arch. f. Schiffs-u. Trop. Hyg. **21**: 306-311, 1917.
7. Vialatte, E. F. C., and Remontet, J. E.: Un cas de purpura provoque par la quinine, Arch. Med. et Pharm. Milit. **91**: 345-349, 1929.
8. Peshkin, M. M., and Miller, J. A.: Quinine and Ergot Allergy and Thrombocytopenic Purpura, J. A. M. A. **102**: 1737-1740, 1934.
9. Beiglboek, W.: Ein Fall von thrombopenischer Purpura bei echter Chininuberempfindlichkeit, Ztschr. klin. Med. **131**: 308-316, 1937.
10. Shrager, J., and Kean, B. H.: Purpura as a Complication of Malaria, Am. J. M. Sc. **212**: 54-59, 1946.
11. Varas, R. P.: Observacion de un caso de purpura hemorrhagica por intoxicacion con sales de quinina, Bol. Soc. Cubana de Pediat. **14**: 49-57, 1942.
12. Chapuis, J. P., and Hemmeler, G.: Quinine et moelle osseuse, Helvet. med. acta **11**: 195-199, 1944.
13. Grandjean, L. C.: A Case of Purpura Hemorrhagica After Administration of Quinine With Specific Thrombocytolysis Demonstrated in vitro, Acta med. scandinav. Supp. **213**: 165-170, 1948.
14. Mamou, H.: Intolérance grave à la quinine guerison par transfusions massives, Rev. du Palud. et de Méd. Trop. **5**: 273, 1947.
15. Bolton, F. G., and Young, R. V.: Observations on Cases of Thrombocytopenic Purpura due to Quinine, Sulphamezathine and Quinidine, J. Clin. Path. **6**: 320-323, 1953.
16. Rappoport, A. E., Nixon, C. E., and Barker, W. A.: Fatal Secondary, Toxic Thrombocytopenic Purpura due to Sodium Salicylate, J. Lab. & Clin. Med. **30**: 916-927, 1945.
17. Dameshek, W.: A Method for the Simultaneous Enumeration of Blood Platelets and Reticulocytes, Arch. Int. Med. **50**: 579-589, 1932.
18. Rees, H. M., and Ecker, E. E.: An Improved Method for Counting Platelets, J. A. M. A. **80**: 621-622, 1923.
19. Harrington, W. J., Sprague, C. C., Minnich, V., Moore, C. V., Ahlvin, R. C., and Dubach, R.: Immunologic Mechanisms in Idiopathic and Neonatal Thrombocytopenic Purpura, Ann. Int. Med. **38**: 433-469, 1953.
20. Bigelow, F. S., and Desforges, J. F.: Platelet Agglutination by an Abnormal Plasma Factor in Thrombocytopenic Purpura With Quinidine Ingestion, Am. J. M. Sc. **224**: 274-280, 1952.
21. Sprague, C. C., Harrington, W. J., Lange, R. D., and Shapleigh, J. B.: Platelet Transfusions and the Pathogenesis of Idiopathic Thrombocytopenic Purpura, J. A. M. A. **150**: 1193-1198, 1952.
22. Hirsch, E. O., and Dameshek, W.: Thrombocytopenic Purpura due to Allergy to Quinidine. Study of the Mechanism of Thrombocytopenia, Am. J. Med. **9**: 828-833, 1950.
23. Moeschlin, S.: Die Sedormid-thrombocytopenie anhand von Sternalpunktaten, Belastungs- und Transfusions-versuchungen, Schweiz. med. Wchnschr. **72**: 119-124, 1942.
24. Moeschlin, S., Meyer, H., Israels, L. G., and Tarr-Gloor, E.: Experimental Agranulocytosis. Its Production Through Leucocytic Agglutination by Antileucocytic Serum, Acta Haemat. **11**: 73-94, 1954.

# THE HEMOLYTIC EFFECT OF PRIMAQUINE

## V. Primaquine Sensitivity as a Manifestation of a Multiple Drug Sensitivity

Raymond J. Dern, M.D., Ph.D.,* Ernest Beutler, M.D.,** and Alf S. Alving, M.D.
Chicago, Ill.

PREVIOUS studies have shown that about 10 per cent of American Negroes have an intrinsic defect of the red blood cell which results in the development of an acute hemolytic anemia whenever they are given 30 mg. of primaquine base daily.[1] Subsequent studies have indicated that because of this defect erythrocytes are also unusually susceptible to hemolysis by a variety of other compounds.

The present report is limited to a consideration of a series of eight aniline derivatives, all established therapeutic agents. These investigations were undertaken to (1) determine which members of this series of compounds were hemolytically active against primaquine-sensitive cells, (2) define the characteristics of the resulting hemolytic anemia when these compounds were administered to primaquine-sensitive men, and (3) study the effects of certain of these drugs on primaquine-nonsensitive cells.

### MATERIALS AND METHODS

All subjects were inmate volunteers from the Illinois State Penitentiary, Stateville Branch, Joliet, Illinois. In this paper the term "normal," when referring either to cell populations or to individuals, implies nonsensitivity to the hemolytic action of 30 mg. of primaquine base administered daily. The dosage of primaquine, sulfanilamide, acetanilid, Phenacetin, thiazolsulfone (Promizole) and para-hydroxyacetanilid (Tralgon) has been expressed in terms of base; phenylhydrazine as the hydrochloride, and sulfoxone (Diasone) and para-aminobenzoic acid as the sodium salt.

$Cr^{51}$-tagged erythrocytes were used for survival studies. The labeling technique was carried out as described in an earlier paper.[1] Corrections for background and decay of the isotope were made before all calculations. In order to facilitate visual comparison of the survival curves, all $Cr^{51}$ counts (C) were expressed as a percentage of the initial count. The survival curves obtained by the $Cr^{51}$ technique are exponential over a period of several weeks. The apparent half-survival time of erythrocytes by this method is shorter than that obtained by the Ashby technique, due to the elution of $Cr^{51}$ from red blood cells in the circulation, taking place at an exponential rate.[2-4] At any time (t), after the initial $Cr^{51}$ count, the total $Cr^{51}$ remaining ($C_t$) is a product of the mean amount of $Cr^{51}$ on each labeled cell ($E_t$) and the total number of labeled cells remaining ($A_t$):

$$(1) \quad C_t = E_t \, A_t$$

---

From the Army Malaria Research Unit, Department of Medicine, The University of Chicago, Chicago, Ill.

This work was supported in part by the Research and Development Division, Office of the Surgeon General, Department of the Army, under contract No. DA-49-007-MD-20 with the Department of Medicine, The University of Chicago, Chicago, Ill.

The investigations reported in this paper would not have been possible except for the cooperation of the inmates and the administrative staff of Stateville Penitentiary.

Received for publication Aug. 16, 1954.

*Captain, MC, USA, 9927-TSU-SGO.

**Lieutenant, MC, USA, 9927-TSU-SGO.

In this study, we have applied the $Cr^{51}$ cell survival study technique to the problem of determining what fraction of erythrocytes were destroyed by the action of the administered drug (Fig. 1). Test drugs were administered for an eight-day period and the degree of hemolysis was measured on the final day of drug ($t_1$ days after the initial $Cr^{51}$ count). In each case, the $Cr^{51}$ count at time $t_1$ ($C_{t_1}$) of the recipient receiving drug was compared to the $Cr^{51}$ count which would have been present had no drug been given ($C'_{t_1}$). This value ($C'_{t_1}$) was obtained by linear extrapolation on semilogarithmic coordinates of the predrug control survival curve. This method is an acceptable procedure because of the very consistent exponential character of the $Cr^{51}$ curves for at least thirty days when no abnormal hemolysis takes place.[1, 2, 4] Since (1) $C_{t_1} = E_{t_1} A_{t_1}$ and (2) $C'_{t_1} = E_{t_1} A'_{t_1}$, where $A'_{t_1}$ is the expected number of cells surviving had no drug been given,

$$(3) \quad \frac{C'_{t_1} - C_{t_1}}{C'_{t_1}} = \frac{A'_{t_1} - A_{t_1}}{A'_{t_1}}$$

by substitution of (1) and (2). $E_{t_1}$, the quantity of $Cr^{51}$ on each cell, cancels out of the substituted equation.

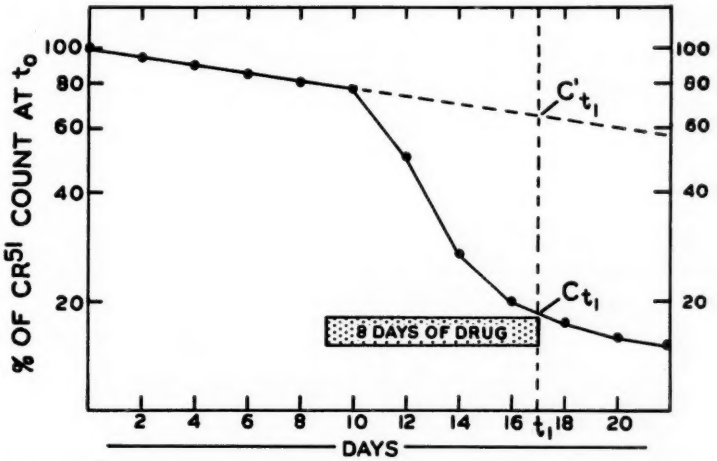



Fig. 1.—$Cr^{51}$ survival curve illustrating the method of measurement of degree of hemolysis. $C'_{t_1}$ is the expected per cent of the initial $Cr^{51}$ count at time $t_1$; $C_{t_1}$ is the actual per cent of $Cr^{51}$ remaining at time $t_1$ (See text, Materials and Methods.) The data is from an experiment in which primaquine was administered to the recipient of sensitive cells.

It is apparent, therefore, that the experimentally observed ratio $\frac{C'_{t_1} - C_{t_1}}{C'_{t_1}}$ is equal to the ratio of the *actual* number of cells destroyed by the action of the drug to the number which would have been present had no drug been given.

It has been shown previously that young erythrocytes are refractory to the hemolytic action of primaquine.[5] There is evidence that this is also true in the case of other compounds to which these cells are sensitive (vide infra). Because of this fact an increasing proportion of the labeled cells are destroyed if the labeled population is allowed to age in the circulation prior to drug administration. It is easily demonstrated that this effect of aging may be removed by multiplying the fraction of cells destroyed through the action of the drug $t_1$ days after the initial sample, $\frac{C'_{t_1} - C_{t_1}}{C'_{t_1}}$ by $\frac{110 - t_1}{t_1}$, assuming a 110-day mean life span and that the value of $t_1$ is sufficiently low so that all cells $t_1$ or less days old are refractory to hemolysis. We have previously shown that the latter condition is fulfilled when $t_1$ is 21 days[5] or less in the case of primaquine. The corrected value,

$$(4) \quad \frac{C'_{t_1} - C_{t_1}}{C'_{t_1}} \cdot \frac{110 - t_1}{110} \cdot 100$$

represents the percentage of the unaged population which is susceptible to hemolysis by

the drug studied. Since in this study the extreme values of $t_1$ at which eight days of drug administration were completed were 15 and 20 days, the magnitude of the correction factor was about the same in all cases.

## RESULTS

### 1. *The Effects of Eight Aniline Derivatives on Primaquine-Sensitive Red Blood Cells.*—

a. *Procedure:* In each experiment, $Cr^{51}$-labeled erythrocytes from either a primaquine-sensitive or a nonsensitive donor were transfused into a group of nonsensitive recipients. After a control period of about ten days, each recipient was given one of the test com-

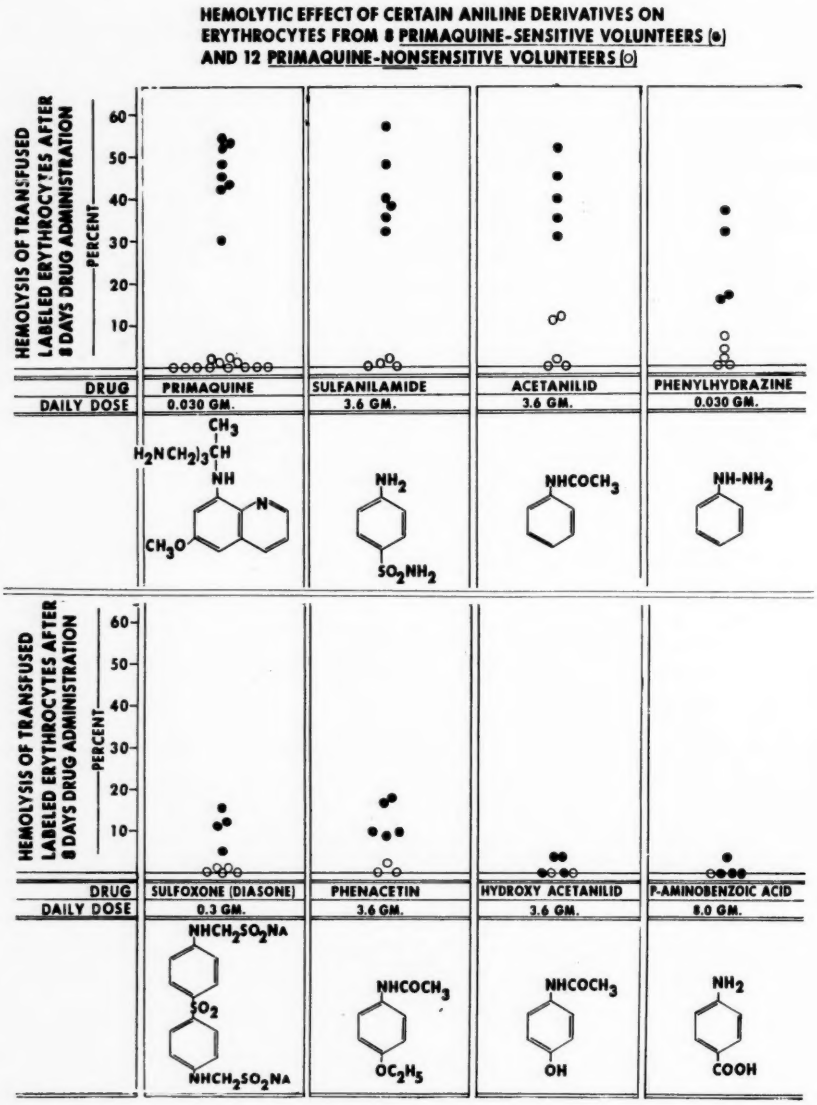



Fig. 2.—The hemolytic effect of primaquine and seven aniline derivatives on primaquine-sensitive and nonsensitive red cells. In each column, each point represents the per cent hemolysis of transfused labeled cells from a *different donor.*

pounds. The drugs were administered in arbitrarily selected doses, but usually in approximately therapeutic amounts. Primaquine, phenylhydrazine, and sulfoxone (Diasone) were given in a single daily dose; the others were given every four hours. All drug programs were continued for at least eight days.

In one group of studies, the erythrocytes of each of three primaquine-sensitive and three primaquine-nonsensitive volunteers were tested against primaquine and against five or more of the eight aniline derivatives. In the remainder of the experiments, erythrocytes from a larger number of sensitive and nonsensitive men were each tested against a smaller number of drugs.

b. *Observations:* The results of studies of the hemolytic effect of primaquine and each of seven aniline derivatives on erythrocytes from eight different primaquine-sensitive donors and twelve different normal (primaquine-nonsensitive) donors are given in Fig 2. Where duplicate determinations were made, only the first value is given in the figure.

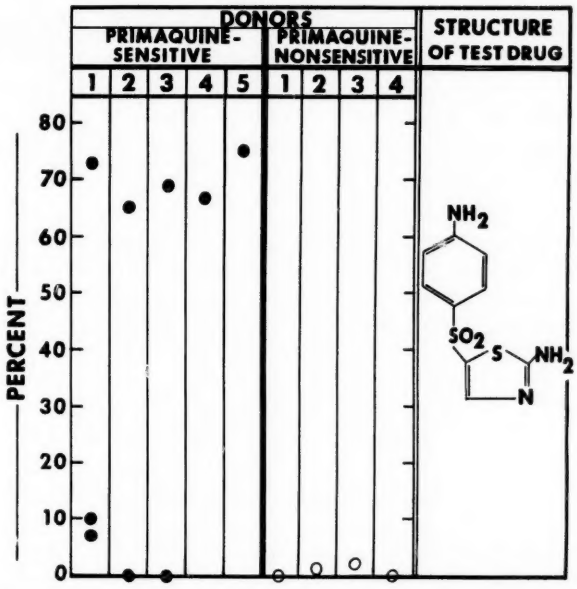



Fig. 3.—The hemolytic effect of thiazolsulfone on labeled transfused erythrocytes from five primaquine-sensitive and four nonsensitive donors. In each column, each point indicates the per cent hemolysis of transfused labeled cells in a different thiazolsulfone-treated recipient.

It is evident that primaquine-sensitive erythrocytes were highly sensitive to the administration of sulfanilamide and acetanilid, whereas normal cells were only slightly, or not at all, affected by these compounds. With phenylhydrazine, the difference between primaquine-sensitive and normal cells was distinct but not as striking as with the other compounds, possibly because of the known variability of response to this drug when orally administered. Sulfoxone (Diasone) and phenacetin were moderately hemolytic to primaquine-sensitive cells in the doses used, but had no effect on normal cells. Parahydroxyacetanilid (Tralgon) and para-aminobenzoic acid did not hemolyze either primaquine-sensitive or normal cells, although rather large doses of each compound were given.

The response to thiazolsulfone (Promizole) was frankly erratic, as shown in Fig. 3. Although normal cells were not destroyed, the hemolytic effect of this compound on primaquine-sensitive cells varied from 0 to 76 per cent. Since erythrocytes from the same primaquine-sensitive donor were hemolyzed to a great extent in one recipient, but not at all in another when thiazolsulfone was administered, it is evident that the observed variability was due to differences in the absorption, excretion, or metabolism of the drug by the individual carriers of the labeled cells. It was observed that the greatest thiazolsulfone-induced destruction of primaquine-sensitive cells occurred in a normal recipient who developed a marked methemoglobinemia, abdominal pain, nausea, headache, and slight anemia during drug administration. This subject, when given only 3.0 Gm. of thiazolsulfone daily, developed severe toxic symptoms, and hemolyzed transfused primaquine-sensitive cells almost as extensively as when 6.0 Gm. were given. On the other hand, sensitive cells from the same donor, when transfused into another recipient, were not hemolyzed by the daily administration of 18.0 Gm. of thiazolsulfone. It was noted that the latter recipient did not exhibit any signs of toxicity. It is evident that primaquine-sensitive cells are highly susceptible to hemolysis by thiazolsulfone, but the observed degree of hemolysis in an individual is dependent on extracorpuscular factors characteristic of the particular recipient.

2. *Characteristics of the Hemolytic Anemia.*—

a. *Procedure:* Certain of the hemolytic compounds were administered directly to primaquine-sensitive men. Two such men were given acetanilid, 3.6 Gm. daily; two others received 3.6 Gm. of sulfanilamide daily. A fifth sensitive man was given phenylhydrazine in doses varying from 15 to 30 mg. daily. Each man received the drug continuously for from three to five weeks. At the end of this period, primaquine in doses of 30 mg. daily was substituted for the test drug. In each case, hemoglobin and reticulocyte count determinations were made daily or every other day throughout the period of drug administration. In four of the cases, an aliquot of the volunteer's erythrocytes, labeled with $Cr^{51}$, was injected into him prior to the experiment.

b. *Observations:* Typical studies of a sulfanilamide- and an acetanilid-induced hemolytic anemia are given in Figs. 4 and 5, respectively. The course of the hemolytic reaction was identical to that of the primaquine-induced anemia which was described in an earlier paper.[6] The onset of the anemia occurred within three to four days after exhibition of the drug, and the hemoglobin reached its lowest values in from eight to ten days. Recovery began shortly thereafter although drug administration was continued. The transient reticulocytosis and darkening of the urine observed during a primaquine-induced hemolysis was also noted in these studies. The substitution of primaquine for the test drug had little or no effect on the pattern of recovery. In the case of the acetanilid-induced hemolysis (Fig. 5), a slight decrease in hemoglobin concentration occurred during the subsequent administration of primaquine. This was probably due to the fact, demonstrated by $Cr^{51}$-labeled red cell survival studies, that the cells of this volunteer were somewhat more sensitive to primaquine than to acetanilid.

In a volunteer who was given small and variable doses of phenylhydrazine over a period of five weeks the hemoglobin level decreased to about 87 per cent of normal and subsequently returned to normal levels while drug admin-

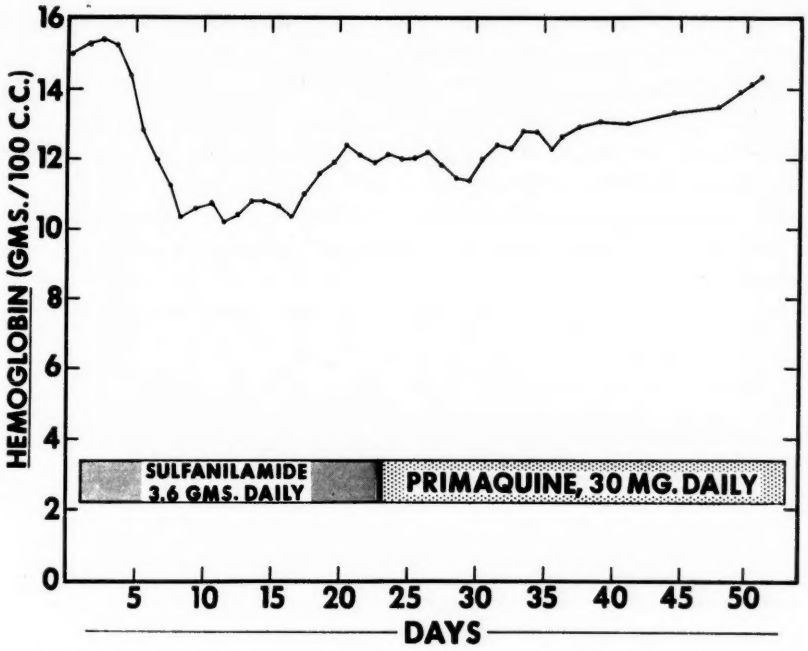



Fig. 4.—Sulfanilamide-induced anemia followed by primaquine insensitivity. The course of the hemolytic anemia resulting from the administration of sulfanilamide to a primaquine-sensitive man.

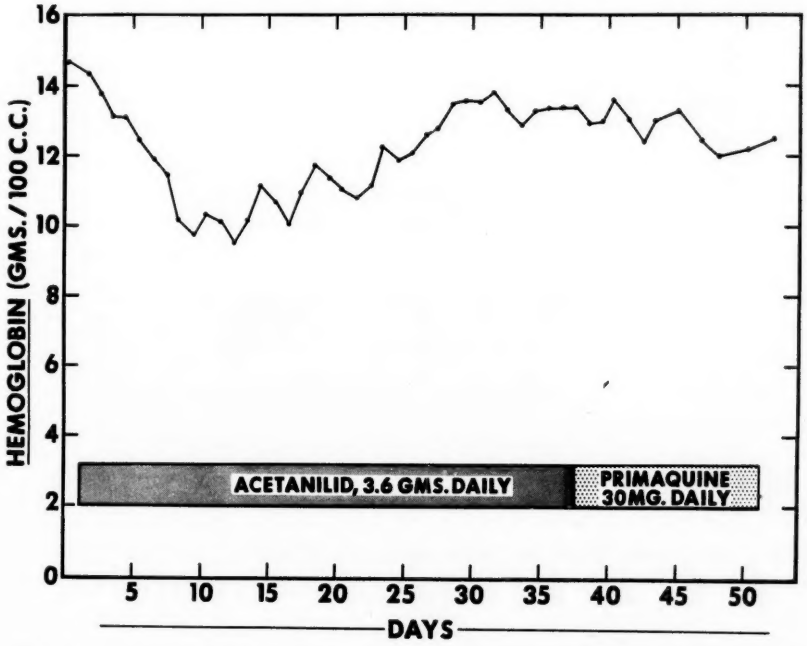



Fig. 5.—Acetanilid-induced anemia followed by primaquine sensitivity. The course of the hemolytic anemia resulting from the administration of acetanilid to a primaquine-sensitive man.

istration was continued. Labeled cell studies carried out simultaneously indicated a moderate hemolysis. When primaquine was substituted for phenylhydrazine there resulted an abrupt decrease in hemoglobin concentration to 81 per cent of normal followed immediately by progressive recovery. Symptoms of acute hemolysis did not occur. However, in other experiments, when primaquine was administered to this man without preadministration of phenylhydrazine, the hemoglobin concentration fell precipitously to 50 per cent of normal, with associated acute symptoms. Definite amelioration of the primaquine-induced hemolytic reaction was produced by the previous administration of phenylhydrazine.

3. *Effects of Certain Aniline Derivatives on Normal Red Blood Cells.*—

a. *Procedure:* In the studies reported in this section, normal cells and individuals were tested with various amounts or with combinations of certain of the compounds. The maximal dose and the duration of administration was kept within the range considered safe in all respects for the particular drug. The response to the administration of drug was measured both by hemoglobin determinations and by $Cr^{51}$-labeled cell studies.

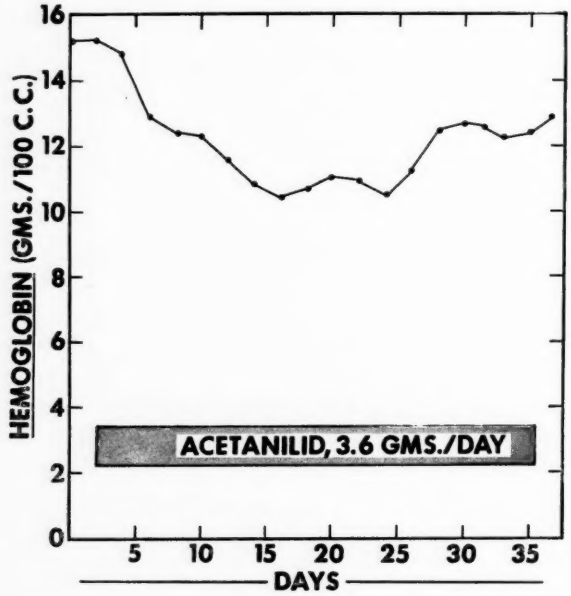



Fig. 6.—The course of an acetanilid-induced hemolytic anemia in a primaquine-nonsensitive man.

b. *Observations:*

(1) Hemolytic effect of large doses of drug on normal cells: When 120 mg. of primaquine was administered daily to each of three normal men for fourteen days, a mild hemolytic anemia was observed in two cases.

When another normal volunteer was give 90 mg. of primaquine together with 3.6 Gm. of acetanilid daily, a marked hemolytic anemia resulted.

The hemolytic effect of large doses of phenylhydrazine on normal cells is well known and was, therefore, not included in this study.

(2) Hemolysis of normal cells by 3.6 Gm. acetanilid daily: The occurrence of a mild hemolysis of two populations of normal cells by acetanilid was indicated in Fig. 2. The degree of hemolysis, however, was slight when compared to the severe drug-induced destruction of primaquine-sensitive cells. It was observed that both of the recipients in whom labeled normal cells were hemolyzed developed toxic manifestations (marked methemoglobinemia, nausea, abdominal cramps, and anemia) during drug administration. In a subsequent study, one of these men was given 3.6 Gm. acetanilid daily for five weeks. The resulting hemolytic anemia is shown in Fig. 6. The pattern of the anemia was similar to that observed when certain of the hemolytically active drugs of the series were administered to sensitive men, but the rate of development of anemia was much slower, the minimum point not being reached until sixteen to eighteen days after beginning the drug. Recovery occurred, however, although drug administration was continued.

## DISCUSSION

It has been shown that primaquine-sensitivity of erythrocytes is consistently associated with a sensitivity to a variety of other compounds and, for this reason, should be considered a manifestation of a multiple drug sensitivity. The characteristics of the hemolytic anemia resulting from the administration of either sulfanilamide or acetanilid to a primaquine-sensitive man are identical to those of a primaquine-induced hemolytic anemia.[6] We believe that the temporary primaquine insensitivity which immediately follows the hemolytic anemia induced by certain aniline derivatives is caused by the elimination of primaquine-sensitive cells by the first compound administered, and, therefore, that the same group of erythrocytes (older cells[5]) is involved in the several hemolytic reactions.

Since primaquine, acetanilid, and phenylhydrazine (and probably the other active compounds) induce hemolysis of normal cells when given in large enough doses, the state of drug nonsensitivity must be evaluated relative to the dose administered. Thus, "sensitivity" should be considered as an unusual susceptibility to hemolysis by this series of generally hemolytic compounds. However, the usual dose range of these drugs permits the clinical classification of cells as "sensitive" or "nonsensitive."

Although the occurrence of a severe acute hemolytic anemia in response to the administration of these drugs depends primarily on the presence of sensitive cells, extracorpuscular factors characteristic of the individual recipient can modify the reaction. This effect is seen in its extreme degree in the variability in response to thiazolsulfone.

It is believed that the daily administration of 3.6 Gm. of acetanilid approaches the maximum tolerated dose of this drug for normal individuals, above which level, according to our concept, hemolysis of normal cells would occur. At this "breaking point" it would be expected that hemolytic anemia would occur in some normal subjects, such as was observed with daily administration of 120 mg. of primaquine. Other toxic symptoms (increased methemoglobin, nausea, and abdominal cramps) appeared in both normal men who developed hemolytic anemia in response to 3.6 Gm. of acetanilid.

Many clinical reports of hemolytic anemia occurring in response to the administration of certain members of this series of compounds have appeared.[7-11] The extensive report of Wood[10] is of particular interest. In his series of 522 cases at Johns Hopkins Hospital, twenty-one patients developed an acute hemolytic anemia on administration of sulfanilamide. Although the ratio of Negro to Caucasian admissions is not known for the year during which the study was made, Wintrobe[12] gives a 1 to 3 ratio for the years 1925 to 1940. If this ratio is applied to Wood's data, the incidence of sulfanilamide-induced hemolytic anemia is about 12 per cent among Negroes and 1.3 per cent among Caucasians. This incidence of sensitivity among Negroes is in close agreement with our estimated value. In over 1,000 Caucasians treated at Stateville with 30 mg. of primaquine daily or the equivalent of other hemolytic 8-aminoquinolines we have observed only one case of acute hemolytic anemia. The higher incidence of hemolysis in Caucasians receiving sulfanilamide, reported by Wood,[10] may be related to the development of unusual extracorpuscular factors such as those described in this paper.

## SUMMARY

Primaquine-sensitive cells were shown to be unusually susceptible to hemolysis by acetanilid, sulfanilamide, thiazolsulfone (Promizole), phenylhydrazine, sulfoxone (Diasone), and phenacetin. In very high doses or in combinations most of the active compounds were shown to be hemolytic to normal cells.

Daily administration of 3.6 Gm. of para-hydroxyacetanilid (Tralgon) or 8.0 Gm. of para-aminobenzoic acid did not produce hemolysis in either primaquine-sensitive or normal cells.

The course of the severe but self-limited hemolytic anemia resulting from the administration of certain of these compounds to sensitive men was identical to that previously described for primaquine-induced hemolytic anemia. Immediately following the administration of these compounds, a temporary primaquine insensitivity was observed.

The probable mechanism of drug-induced hemolytic anemia seen occasionally in normal men is described.

The relationship of this red cell abnormality and certain modifying extracorpuscular factors to reported clinical observations of drug toxicity is discussed.

## REFERENCES

1. Dern, R. J., Weinstein, I. M., LeRoy, G. V., Talmage, D. W., and Alving, Alf S.: The Hemolytic Effect of Primaquine. I. The Localization of the Drug-Induced Hemolytic Defect in Primaquine-Sensitive Individuals, J. LAB. & CLIN. MED. **43**: 303-309, 1954.
2. Necheles, T. F., Weinstein, I. M., and LeRoy, G. V.: Radioactive Sodium Chromate for the Study of Survival of Red Blood Cells. I. The Effect of Radioactive Sodium Chromate on Red Cells, J. LAB. & CLIN. MED. **42**: 356-367, 1953.
3. Ebaugh, F. G., Jr., Emerson, C. P., and Ross, J. F.: The Use of Radioactive Chromium$^{51}$ as an Erythrocyte-Tagging Agent for the Determination of Red Cell Survival in vivo, J. Clin. Invest. **32**: 1260-1276, 1953.
4. Read, R. C., Wilson, G. V., and Gardner, F. H.: The Use of Radioactive Sodium Chromate to Evaluate the Life Span of the Red Blood Cell in Health and Certain Hematologic Disorders, Am. J. Med. Sc. **228**: 40-52, 1954.

5. Beutler, E., Dern, R. J., and Alving, Alf S.: The Hemolytic Effect of Primaquine. IV. The Relationship of Cell Age to Hemolysis, J. LAB. & CLIN. MED. **44**: 439-442, 1954.
6. Dern, R. J., Beutler, E., and Alving, Alf S.: The Hemolytic Effect of Primaquine. II. The Natural Course of the Hemolytic Anemia and the Mechanism of Its Self-limited Character, J. LAB. & CLIN. MED. **44**: 171-176, 1954.
7. Gross, M.: Acetanilid, New Haven, 1946, Hillhouse Press.
8. Cornbleet, T.: Sulfoxone (Diasone) Sodium for Dermatitis Herpetiformis, Arch. Dermat. & Syph. **64**: 684-687, 1951.
9. Jasinski, B., and Muller, D.: Zur Pathegenese der toxischehamolytischen Anamien nach chronischem Phenacetinabusus, Schweiz. Med. Wchnschr. **80**: 681 and 1113, 1950.
10. Wood, W. B., Jr.: Anemia During Sulfanilamide Therapy, J. A. M. A. **11**: 1916-1919, 1938.
11. Meyler, L.: Side Effects of Drug, New York, 1952, Elsevier Press, Inc.
12. Wintrobe, M.: Clinical Hematology, Philadelphia, 1951, Lea & Febiger, p. 434.

# THE HEMOLYTIC EFFECT OF PRIMAQUINE

## VI. An in Vitro Test for Sensitivity of Erythrocytes to Primaquine

Ernest Beutler, M.D.,* Raymond J. Dern, M.D., Ph.D.,**
and Alf S. Alving, M.D.
Chicago, Ill.

WHEN primaquine is administered to sensitive men an acute hemolytic anemia develops.[1, 2] This unusual sensitivity has been shown to be due to an intrinsic "defect" of the erythrocytes.[2] Recent studies have also shown that these erythrocytes are not only unusually sensitive to the administration of primaquine, but also to phenylhydrazine, acetanilid, sulfanilamide, Phenacetin, thiazolsufone, and certain related compounds.[3]

Our experience in the administration of primaquine to 230 Negro volunteers indicates that, although there are varying degrees of intensity of the hemolytic reaction among sensitive individuals, the unusual sensitivity of these erythrocytes does not merely represent one extreme of a continuous, normal sensitivity distribution curve.[3, 4] Rather, a fairly marked degree of sensitivity is present or the cells are completely insensitive. Sensitive erythrocytes, therefore, appear to constitute a distinct group.

This "blood group" cannot be identified morphologically, immunologically, with respect to hemoglobin type, or with respect to fragility to a variety of agents.[5] Until now, only two methods have been available to determine whether or not the erythrocytes of an individual are sensitive. Primaquine may be administered to the individual and his blood and urine observed for evidence of acute hemolysis, or the survival of the erythrocytes may be measured after transfusing them into a recipient who is given primaquine. Both of these methods are obviously tedious and subject a human volunteer to a certain risk. They are therefore completely unsuitable for clinical use or for studies of the incidence and genetics of the sensitive group. For these purposes it has been necessary to develop a simple in vitro test which will accurately identify sensitive cells.

In a previous study we demonstrated that, when primaquine was administered, the extent to which Heinz bodies were formed in the blood of sensitive men was considerably greater than the extent to which they were formed in the blood of nonsensitive men.[5] These bodies are believed to consist of denatured proteins and are a manifestation of red cell damage.[6] A number of agents will cause formation of Heinz bodies under suitable in vitro conditions.[6]

---

From the Army Malaria Research Unit, Department of Medicine, The University of Chicago, Chicago, Ill.

This work was supported in part by the Research and Development Division, Office of the Surgeon General, Department of the Army, under contract No. DA-49-007-MD-20 with the Department of Medicine, The University of Chicago, Chicago, Ill.

The investigations reported in this paper would not have been possible except for the cooperation of the inmates and the administrative staff of Stateville Penitentiary.

Received for publication Aug. 16, 1954.

*Lieutenant, MC, USA, 9927-TSU-SGO.

**Captain, MC, USA, 9927-TSU-SGO.

After preliminary observations indicated that primaquine-sensitive erythrocytes were more susceptible to in vitro as well as to in vivo Heinz body formation than were nonsensitive cells, we developed the standardized test procedure which is reported here. It is a simple laboratory test, based on the in vitro formation of Heinz bodies, which is designed to make it possible to determine whether the individual whose blood is tested is primaquine-sensitive. By inference, from the studies of Dern and associates,[3] the test also makes it possible to detect unusual sensitivity to the hemolytic action of sulfanilamide, acetanild, thiazolsulfone, and certain other drugs.

## MATERIALS AND METHODS

A. *Test Subjects.*—The selection of subjects has been described previously.[2] The tests reported in this study were performed on blood samples from all of the 88 Negro volunteers who received their first course of 30 mg. of primaquine daily at any time within the past nine months. In addition, blood samples from 16 Caucasians, all of whom had been shown to be nonsensitive, were examined. All volunteers were given 30 mg. primaquine daily for at least seven days, the majority for fourteen days. They were then classified as either "sensitive" or "nonsensitive" on the basis of the occurrence or absence of clinical evidence of acute hemolysis. In 14 subjects, including the two in whom false positive results were obtained with the "standard test procedure" described below, cell survival studies were also carried out by the $Cr^{51}$ technique[2] during primaquine administration. In each of these cases, the results of the cell survival studies confirmed the clinical impression. Of the 88 Negro volunteers given primaquine in this study, 18 were primaquine-sensitive and 70 were nonsensitive. In 81 cases the test was carried out seven days to seven months after the course of primaquine had been administered; in 23 cases, including five sensitive men, the test was performed before primaquine had been administered.

B. *Materials.*—

1. *Blood samples:* Three to ten milliliter blood samples were drawn into heparinized syringes and transferred into screw cap vials 24 mm. in diameter. Before use the samples were mixed by gently agitating with a rotary motion. All blood samples were used within one hour of the time they were drawn.

2. *Buffer solution:* A buffer solution at pH 7.6 was prepared by mixing 1.3 parts M/15 $KH_2PO_4$ with 8.7 parts of M/15 $Na_2HPO_4$. Sufficient glucose was added to make a final concentration of 200 mg. per cent. This solution is stable in the refrigerator for a considerable period of time but growth of molds occasionally necessitates preparation of a new solution.

3. *Acetylphenylhydrazine solution:* A 100 mg. per cent acetylphenylhydrazine solution was prepared by dissolving powdered acetylphenylhydrazine (C. P., Fisher) in buffer solution at room temperature. The acetylphenylhydrazine solution was used within one hour after preparation except where otherwise indicated.

4. *Crystal violet solution:* Approximately 2.0 Gm. of crystal violet (C.I. 681, National Aniline, Certified) were added to 100 ml. of an aqueous 0.73 per cent NaCl solution at room temperature. The mixture was shaken for five minutes to aid solution and then filtered. The filtrate was mixed with an equal quantity of the 0.73 per cent NaCl solution. We have found the dye solution to be stable at room temperature for a period of at least five months.

C. *Standard Test Procedure.*—With a 0.1 ml. "blowout" pipette, 0.1 ml. of whole blood was added to 2 ml. of acetylphenylhydrazine solution in a test tube (inner diameter 12 mm.). The suspension was immediately mixed and aerated two or three times by drawing approximately 0.1 ml. into the pipette and blowing it, and a small quantity of air, back into the suspension. The pipette was left in the uncapped tube, which was placed in a 37° C. water bath. After two hours the cell suspension was agitated and aerated by blowing briefly through the pipette. Two hours later the suspension was again agitated in the same fashion and a

small drop (approximately 0.01 ml.) placed on a cover slip. The cover slip was then inverted onto a microscope slide on which a larger drop (approximately 0.025 ml.) of the crystal violet solution had been placed. After five to ten minutes a field was selected for oil immersion examination. Certain portions of the slide were generally unsatisfactory for examination because of hemolysis. In such areas only sphered ghosts were seen, the cell membrane taking a deep blue or violet stain. Fields in which the erythrocytes were crenated or in which the cells overlapped were avoided. Rather, fields in which the cells maintained their normal shape and in which there was little overlapping of cells were selected for examination. For quantitative appraisal of the results, counts of one or two hundred erythrocytes were made to determine the percentage containing five or more Heinz bodies. This number of Heinz bodies was chosen somewhat arbitrarily on the basis of preliminary experiments, and subsequently proved to be quite satisfactory for our purposes. When Heinz bodies were present in clusters, as many separate granules as could be clearly distinguished were counted. The percentage of cells forming five or more Heinz bodies will subsequently be referred to as the "test result."

## EXPERIMENTAL AND RESULTS

A. *Differentiation of Sensitive From Nonsensitive Erythrocytes by Standard Test Procedure.—*

1. *Appearance and distribution of Heinz bodies:* The differences between the appearance of the test preparations from sensitive and nonsensitive subjects were almost always sufficiently great to permit them to be distinguished without counts being made (Fig. 1). *Sensitive* erythrocytes only occasionally formed solitary, marginal, well-rounded Heinz bodies under the test conditions. Most of the erythrocytes contained many small Heinz bodies. *Nonsensitive* erythrocytes generally formed one large, well-rounded Heinz body, 1 to 2 $\mu$ in diameter, situated at the margin of the cell. A few smaller Heinz bodies were frequently also present, but the total number of Heinz bodies per cell, as indicated by the quantitative appraisal, did not often exceed four.

2. *Quantitative appraisal:*

a. *Single blood samples from 104 individuals (Fig. 2):* Blood from 104 volunteers, 18 of whom were primaquine-sensitive and 86 of whom were nonsensitive, were examined by the standard test procedure (See Methods). When more than one test was performed on a subject for any reason, only the first test result obtained is presented in Fig. 2. In samples of blood from the sensitive men tested, the percentage of erythrocytes developing five or more Heinz bodies varied from 45 to 92 with a mean of 67.8 per cent, and a sample standard deviation of 15.9 per cent. In sharp contrast, the percentage of erythrocytes in blood samples from nonsensitive men developing five or more Heinz bodies varied from 0 to 28 in all but two cases, in whom the percentage was 46 and 62. The mean of all test results on the nonsensitive men was only 11.9 per cent and the sample standard deviation was 9.3 per cent. Therefore, interpretation of the results on the basis of a dividing line at 32.5 per cent, 2.2 standard deviations from the mean of each group, gives no false negatives and only two false positives.

b. *Variability in the samples from a single individual:* The standard test procedure was applied repeatedly to blood samples from a sensitive and a nonsensitive individual over a nineteen-day period. The average of nine test

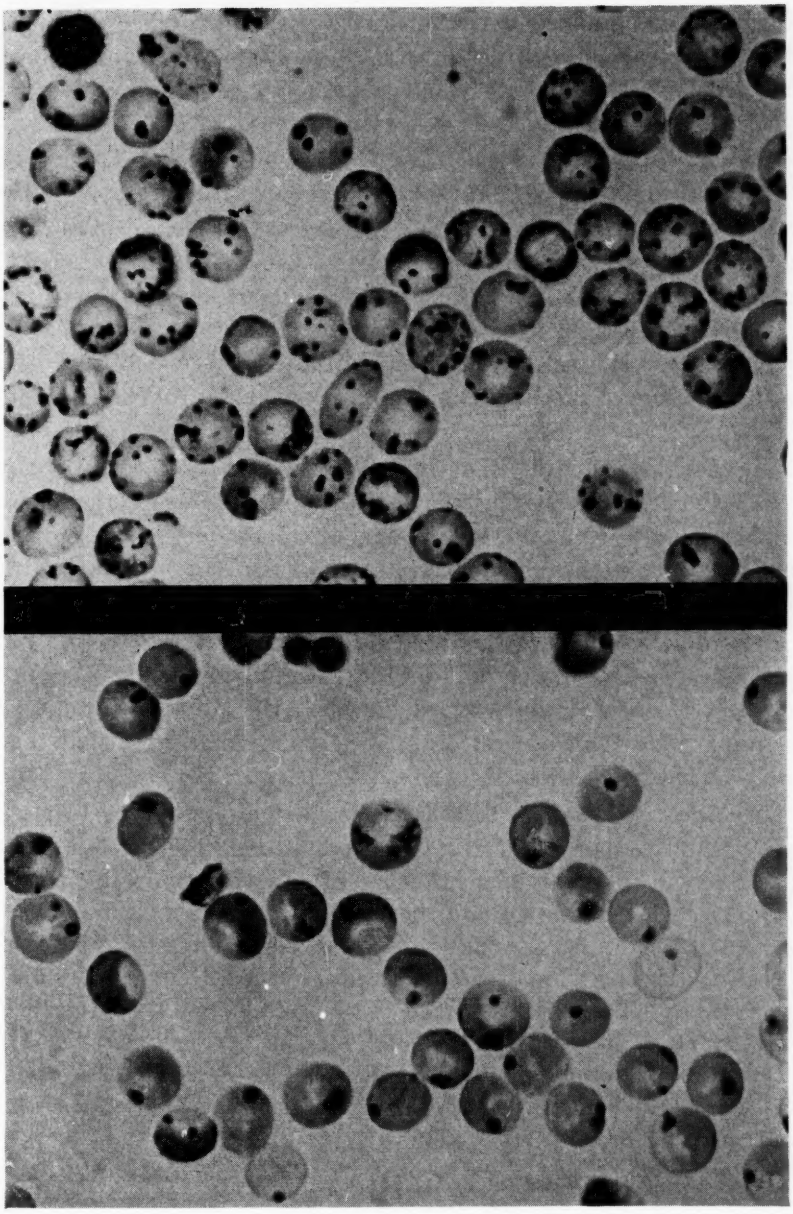


Fig. 1.—Heinz body formation in sensitive (upper field) and nonsensitive (lower field) erythrocytes after incubation with 100 mg. per cent acetylphenylhydrazine solution for four hours ("standard test procedure"). Wet preparations stained with crystal violet. (Magnification ×1,300.)

results on the sensitive cells varied from 42 to 91 per cent, with a mean of 70 per cent, and a sample standard deviation of 16.3 per cent; the ten test results on the nonsensitive cells varied from 8 to 20 per cent with a mean of 13.9 per cent and a sample standard deviation of 3.6 per cent. Thus, although there was considerable variability in the test results obtained on the blood of these individuals from day to day, all results remained within the range established for sensitive and nonsensitive cells, respectively.

Repeated tests were also performed on the blood of the two volunteers on whom false positive results had been obtained. These also varied widely, but partly within a range intermediate between clear-cut positive and clear-cut negative results. In the case of the nonsensitive subject whose blood initially gave test results of 62 per cent, the results of six subsequent examinations varied from 21 to 53 per cent, with a mean of 34.5 per cent; in the case of the nonsensitive subject whose blood initially gave test results of 46 per cent, the results of five subsequent examinations varied from 34 to 68 per cent, with a mean of 52.6 per cent.

c. Relationship of degree of hemolysis to the test results: There were, of course, individual differences in the intensity of the hemolytic response of sensitive individuals given 30 mg. of primaquine base daily. These differences did not, however, appear to be correlated with the degree to which the test results of sensitive cells were positive.

To determine whether those clinically nonsensitive men whose red cells gave the highest test results actually underwent subclinical degrees of hemolysis, $Cr^{51}$ survival curves were recorded during the administration of 30 mg. of primaquine daily for fourteen days. The curves indicated that there was no hemolysis whatsoever of even these nonsensitive cells.

B. *The Effect of Various Abnormal Hematological States.*—The standard test procedure or, in some cases, an earlier modification of the procedure, was applied to the blood of individuals suffering from a variety of abnormal hematological states. The erythrocytes of patients with Hodgkin's disease, multiple myeloma, thallasemia, paroxysmal nocturnal hemoglobinuria, polycythemia rubra vera, and hemoglobin C-S disease, all formed Heinz bodies like nonsensitive cells. Results with the blood of one patient with sickle cell disease were unsatisfactory because of distortion of the erythrocytes. One of the subjects who gave a false positive test had sickle cell trait; on the other hand, the cells of other primaquine-nonsensitive and sensitive patients with sickle cell trait reacted in the expected fashion.

C. *Modifying Factors.*—

1. *Composition of buffer solution:* The presence of potassium, phosphate, or glucose was not essential for satisfactory test results. When potassium ion was substituted for all of the sodium ion in the buffer, hemolysis of the cell suspension occurred. The presence of bicarbonate, chloride, or calcium ions did not interfere with the test. A pH of 7.6 was found to be most satisfactory for purposes of this test; increases in the pH from this value caused greater

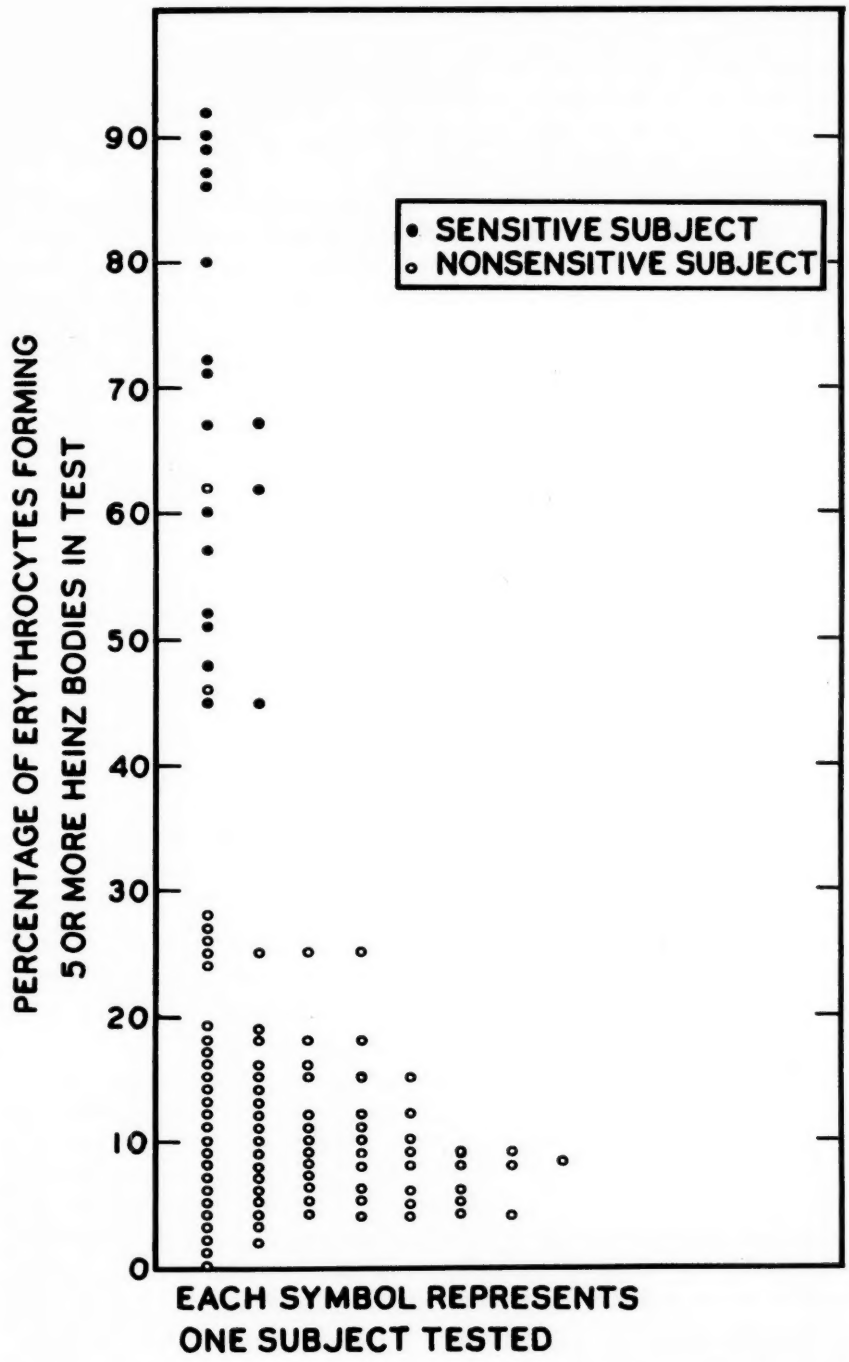



Fig. 2.—The results of tests performed on erythrocytes from eighteen sensitive and eighty-six nonsensitive individuals.

speed of formation, numbers, and size of the Heinz bodies; decreases caused Heinz body formation to be less pronounced and the Heinz bodies to be somewhat scattered even in blood samples from nonsensitive individuals.

2. *Concentration of acetylphenylhydrazine:* The number, size, and speed of formation of Heinz bodies increased with increasing concentrations of acetylphenylhydrazine.

3. *Age of the solution:* The acetylphenylhydrazine solution was somewhat unstable. Solutions kept at 4° C., or at 22° C. for up to seven hours were moderately satisfactory, but tended to result in somewhat weakened Heinz body formation, especially, after over three hours' storage. After twenty-four hours' storage at 4° C., test results were totally unsatisfactory: false positive results were obtained with all samples of nonsensitive cells tested.

4. *Quantity of blood added:* Reducing the quantity of blood added to less than 0.1 ml. per 2 ml. of acetylphenylhydrazine solution increased Heinz body formation; conversely, increasing the amount to more than 0.1 ml. per 2 ml. of acetylphenylhydrazine solution decreased Heinz body formation. Within a range of 0.08 to 0.12 ml. blood per 2 ml. acetylphenylhydrazine solution, however, results of the test remained satisfactory. Parallel changes in test results were observed when the hematocrit was artificially changed by adding to or removing plasma from the blood sample. In one trial, for example, reducing the hematocrit by 50 per cent, from 46 to 23 per cent, increased the test results from 19 to 48 per cent. It is obvious, then, that the hematocrit of very anemic bloods must be adjusted to near normal values by removing plasma if the standards derived from normal blood samples are to be applied.

5. *Incubation temperature:* No Heinz body formation occurred at 4° C. in twenty-four hours. At 22° C. Heinz bodies were seen in a few cells after four hours' incubation, and in all cells after twenty-four hours. Even after twenty-four hours, however, the development of Heinz bodies at 22° C. did not equal that after two or three hours' incubation at 37° C.

6. *Time:* Heinz bodies were first seen in erythrocytes after about one-half hour's incubation with 100 mg. per cent acetylphenylhydrazine. They increased progressively in size and number. Optimal differentiation between sensitive and nonsensitive cells occurred between three and five hours.

7. *Oxygenation:* The extent to which the red cells were oxygenated profoundly influenced the test results. The following experiment illustrates the influence of oxygen on Heinz body formation. Two and five-tenths milliliters of venous blood were drawn under oil and added to 50 ml. of acetylphenylhydrazine solution, also under oil. Carbon monoxide, nitrogen, nitrogen-carbon dioxide, or air were mixed with aliquots of the cell suspension as described in Table I. The test results given in Table I indicate the extent to which Heinz body formation was affected by treatment resulting in varying degrees of oxygen saturation. Heinz body formation was very scanty in the absence of oxygen, while in the oxygen-satured suspension it was very profuse.

It is not surprising, in view of these results, that when capillary blood was used for the test, Heinz body formation was considerably greater than when the standard test procedure, using venous blood, was employed. As has been shown, a satisfactory dividing line between test results with sensitive and

nonsensitive cells when the standard procedure is used is 32.5 per cent. When 0.02 ml. of capillary blood was added to 0.5 ml. of acetylphenylhydrazine solution, the other details of the test being the same, the dividing line between sensitive and nonsensitive cells was increased to between 80 and 85 per cent.

TABLE I. THE EFFECT OF OXYGENATION ON HEINZ BODY FORMATION IN ERYTHROCYTES FROM A SINGLE SENSITIVE AND A SINGLE NONSENSITIVE SUBJECT

| SUSPENSION UNDER: | TREATMENT OF SUSPENSION BEFORE INCUBATION | METHOD OF AGITATION DURING 4-HOUR INCUBATION PERIOD | TEST RESULTS (PER CENT OF CELLS WITH 5 OR MORE HEINZ BODIES) | |
|---|---|---|---|---|
| | | | NONSENSITIVE SUBJECT | SENSITIVE SUBJECT |
| Air | Standard aeration* | Standard aeration* at two hours | 21 | 52 |
| | Shaken with large volume of air for 10 minutes | Standard aerations* every 10 minutes | 85 | 100 |
| Oil | Standard aeration* | Standard aeration* at 2 hours | 13 | 41 |
| | Standard aeration* | Stirred† at 2 hours | 10 | 37 |
| | Standard aeration* | Standard aerations* every 10 minutes | 40 | 99 |
| | Shaken with large volume of air for 10 minutes (without oil) | Stirred† at 2 hours | 73 | 100 |
| | Carbon monoxide passed through suspension for 10 minutes | Stirred† at 2 hours | 0 | 0 |
| | Nitrogen passed through suspension for 10 minutes | Stirred† at 2 hours | 2 | 15 |
| | 94% nitrogen-6% carbon dioxide passed through suspension for 10 minutes | Stirred† at 2 hours | 3 | 0 |

*The standard aeration consisted of briefly blowing into the suspension two or three times through the pipette.

†Without allowing suspension to contact the air.

When very small quantities of venous blood (0.25 to 0.50 ml.) were transferred into 24 mm. screw cap vials instead of the standard 3 to 10 ml. quantities, the details of the test procedure being otherwise unaltered, enhanced Heinz body formation was also observed. This was undoubtedly due to the increased ratio of exposed surface of the blood to its total volume, resulting in more complete oxygenation.

D. *In Vitro Heinz Body Formation in Sensitive and Nonsensitive Erythrocytes by Other Compounds.*—

1. *Phenylhydrazine:* It was possible to distinguish sensitive from nonsensitive cells with a fair degree of accuracy by incubating them with 10 mg. of phenylhydrazine hydrochloride per 100 ml. of the buffer solution for four hours. The number of Heinz bodies formed at this concentration of phenylhydrazine was somewhat greater than that observed with the standard test procedure, and the Heinz bodies were somewhat smaller.

2. *Primaquine:* Heinz body formation by concentrations of primaquine than with acetylphenylhydrazine. It was difficult to differentiate sensitive and nonsensitive cells by the use of this compound, although, in general, more up to 100 mg. per cent in the buffer solution was much less rapid and less marked marked Heinz body formation was observed in the sensitive cells.

3. *Hydoxylamine:* The formation of Heinz bodies by hydroxylamine hydrochloride (Fisher) was very similar to that by acetylphenylhydrazine. Concentrations of only 4 mg. per cent of this substance caused Heinz body formation only slightly less in degree than that observed with 100 mg. per cent acetylphenylhydrazine. Sensitive and nonsensitive cells were well differentiated.

4. *Ascorbic acid:* Ascorbic acid solution, with the pH adjusted to 7.6, caused ample Heinz body formation in concentrations as low as 1.0 mg. per cent. Heinz body formation was more marked in sensitive than in nonsensitive cells. Because of its lack of structural similarity to the other compounds tested, the effect of oxygen on Heinz body formation by ascorbic acid was also investigated. As with acetylphenylhydrazine, Heinz body formation increased with an increased supply of oxygen.

## DISCUSSION

We have demonstrated that erythrocytes of sensitive individuals are more susceptible to the in vitro Heinz body-forming action of a variety of agents than are nonsensitive cells. This has been made the basis of a simple in vitro test which predicts primaquine-sensitivity. In a group of 18 sensitive men tested, there were no false negatives; in a group of 86 nonsensitive men tested, there were two false positives. These have not yet been satisfactorily explained, but were sufficiently few in number so as not to detract materially from the utility of the test.

Although the test results successfully differentiated sensitive from nonsensitive erythrocytes in over 98 per cent of the cases, they could not distinguish between the slight varying degrees of sensitivity which are clinically observed among sensitive individuals. This was to be expected, since the variability observed in the test results on blood samples drawn from a given individual from day to day approximated the variability of the entire group of subjects tested. We do not know whether this day to day variability was due to actual changes in the susceptibility of the erythrocytes, as caused by undetermined external influences, or whether the variability in test results should be ascribed to technical factors which are difficult to control, such as degree of oxygenation and the hematocrit of the blood samples. Undoubtedly, more uniform test results could be obtained by adopting a means of maintaining a constant degree of oxygenation of the cell suspension during the test procedure and by adjusting the hematocrit to a standard value. Although the results would be made more precise, the technical difficulties of the otherwise simple test procedure would greatly increase.

In a previous study[5] it was demonstrated that large numbers of Heinz bodies were formed in vivo in erythrocytes of sensitive men when primaquine was administered. As hemolysis progressed, these Heinz bodies disappeared. On the other hand, only rare Heinz bodies were produced in the erythrocytes of nonsensitive men. It was also shown, in a later study,[7] that young cells, which are known to be relatively resistant to Heinz body formation, were also resistant to hemolysis by primaquine. These findings strongly suggest a "cause and effect" relationship between Heinz bodies and hemolysis. The

demonstration, in the present study, of the increased susceptibility of sensitive cells to in vitro Heinz body formation not only provides a method of determining the presence or absence of sensitivity to primaquine in vitro, but also helps to establish even more firmly the relationship between Heinz bodies and hemolysis.

Thus, it appears that the key to the understanding of the mechanism of hemolysis by this group of compounds is an understanding of the mechanism of Heinz body formation, and the means by which the erythrocyte may protect itself against this process. As Webster's valuable review[6] indicates, the mode of formation of Heinz bodies has been studied extensively, but little agreement has resulted. Warburg and associates[8] suggested that insoluble material formed by the action of phenylhydrazine in blood was denatured globin, derived from hemoglobin. This work was criticized by Kiese and Seipelt,[9] who cited evidence that Heinz bodies are derived from denatured fibroproteins of the erythrocyte stroma.

Regardless of which of these views is correct, the marked inhibition of Heinz body formation which we observed in the absence of oxygen suggested that oxidation plays an important role in the process. It may be that Heinz body-forming compounds act as oxidation-reduction "carriers," being oxidized by oxygen, then oxidizing a red cell component, being reduced in the process, and subsequently being reoxidized by more oxygen. This cycle could continue as long as oxygen was available. Such a mechanism has been suggested previously to explain methemoglobin formation by aryl amines.[10]

On the other hand, it has also been proposed that ascorbic acid and hemoglobin undergo coupled oxidation resulting in formation of oxidized ascorbic acid, and through a peroxide intermediary, choleglobin.[11] Choleglobin is an irreversible hemoglobin breakdown product which is also said to be formed by the action of phenylhydrazine on red blood cells. If Heinz body-forming drugs other than ascorbic acid can similarly undergo coupled oxidation with hemoglobin, this mechanism might also account for their Heinz body-forming properties.

We have previously shown that susceptibility to hemolysis is greatest among the older members of sensitive red cell populations.[7] Unpublished observations have indicated no difference in permeability of sensitive and nonsensitive erythrocytes to hemolytic compounds. It, therefore, appeared likely that a deficiency in one of the enzyme systems of the red cell may be responsible for unusual sensitivity to primaquine. It is known, for example, that diphasphopyridine nucleatide, reduced in the course of red cell glycolysis, plays a part in the reduction of methemoglobin to oxyhemoglobin.[10, 12] It appeared possible that this or other enzyme systems of the red cell similarly protect the hemoglobin or the stroma from the more profound oxidative changes leading to Heinz body formation. Investigation of some of the important enzyme systems of sensitive and nonsensitive red cells have indicated that a deficiency of red cell sulfhydryl compounds is closely associated with sensitivity to hemolysis. These studies will be reported in detail in a later paper.

SUMMARY

The erythrocytes of individuals sensitive to the hemolytic action of primaquine constitute a distinct "group." These erythrocytes form Heinz bodies more readily than do nonsensitive red cells, both under in vivo and under in vitro conditions. A standardized test procedure, based on the difference in in vitro Heinz body formation by acetylphenylhydrazine, is described. Tests of the blood of 104 volunteers, 18 sensitive and 86 nonsensitive, indicated a degree of accuracy exceeding 98 per cent in predicting primaquine sensitivity. Negative test results were obtained on the blood of patients with Hodgkin's disease, multiple myeloma, thallasemia, paroxysmal nocturnal hemoglobinuria, polycythemia rubra vera, and hemoglobin C-S disease. The influence of the composition and age of the buffered acetylphenylhydrazine solution, quantity of blood added, incubation time and temperature, and degree of oxygenation are all described. The degree of oxygenation, in particular, was shown to markedly affect Heinz body formation, oxygen promoting Heinz body formation. Heinz body formation by phenylhydrazine, primaquine, hydroxylamine, and ascorbic acid in sensitive and nonsensitive cells was also compared. The mechanism of Heinz body formation by this group of substances may be oxidation of a red cell component. Sensitive cells may be deficient in a protective enzyme system.

REFERENCES

1. Hockwald, R. S., Arnold, J., Clayman, C. B., and Alving, Alf S.: Status of Primaquine. 4.Toxicity of Primaquine in Negroes, J. A. M. A. **149**: 1568-1570, 1952.
2. Dern, R. J., Weinstein, I. M., LeRoy, G. V., Talmage, D. W., and Alving, Alf S.: The Hemolytic Effect of Primaquine. I. The Localization of the Drug-Induced Hemolytic Defect in Primaquine-Sensitive Individuals, J. Lab. & Clin. Med. **43**: 303-309, 1954.
3. Dern, R. J., Beutler, E., and Alving, Alf S.: The Hemolytic Effect of Primaquine. V. Primaquine-Sensitivity as a Manifestation of Multiple Drug Sensitivity, J. Lab. & Clin. Med. **44**: 30-39, 1955.
4. Beutler, E., Dern, R. J., and Alving, Alf S.: Unpublished observations.
5. Beutler, E., Dern, R. J., and Alving, Alf S.: The Hemolytic Effect of Primaquine. III. A Study of Primaquine-Sensitive Erythrocytes, J. Lab. & Clin. Med. **44**: 177-184, 1954.
6. Webster, S. H.: Heinz Body Phenomenon in Erythrocytes, Blood **4**: 479-497, 1949.
7. Beutler, E., Dern, R. J., and Alving, Alf S.: The Hemolytic Effect of Primaquine. IV. The Relationship of Cell Age to Hemolysis, J. Lab. & Clin. Med. **44**: 439-442, 1952.
8. Warburg, O., Kubowitz, F., and Christian, W.: Ueber die Wirkung von Phenylhydrazin und Phenylhydroxylamin auf den Stoffwechsel der Roten Blutzellen (Methode zur Messung des Stoffwechsels roter Blutzellen), Biochem. Ztschr. **242**: 170-205, 1931.
9. Kiese, M., and Seipelt, L.: Bildung und Elimination von Verdoglobinen, Arch. exper. Path. u. Pharmakol. **200**: 648-683, 1943.
10. Bodanski, O.: Methemoglobinemia and Methemoglobin-Producing Compounds, Pharmacol. Rev. **3**: 144-196, 1951.
11. Lemberg, R., and Legge, J. W.: Hematin Compounds and Bile Pigments, New York, 1949, Interscience Publishers, Inc., p. 476.
12. Bartlett, G. R., and Marlow, A. A.: Enzyme Systems in the Red Blood Cell, Bulletin of the Scripps Metabolic Clinic, San Diego, Calif. **2**: No. 5, 1951.

# THE PERSISTENCE IN THE BLOOD OF THE RADIOACTIVE LABEL OF ALBUMINS, GAMMA GLOBULINS, AND GLOBULINS OF INTERMEDIATE MOBILITY

## II. Comparison of Gamma Globulins Labeled With $S^{35}$ and $I^{131}$ in the Same Subjects

S. H. Armstrong, Jr., M.D.,* John Kukral, M.S., M.D.,**
Jerome Hershman, M.S.,*** Katharine McLeod, M.S.,
Janet Wolter, M.D.,**** and David Bronsky, M.D.
Chicago, Ill.

### INTRODUCTION

IN THE first communication of this series,[1] we reported, among other findings, apparent turnover times for intravenously injected $I^{131}$ labeled human-serum albumins considerably shorter than apparent turnover times of the serum albumins synthesized by the same human subjects following oral ingestion of $S^{35}$ labeled yeast. The $I^{131}$ values were of the order of magnitude reported by others employing intravenous albumin so labeled; the $S^{35}$ values were either as long or longer than the values first reported by London[2] for albumins synthesized following oral ingestion of the $N_{15}$ label.

This finding, together with the studies of others on preparations of $I^{131}$ labeled albumins both in vivo and with the ultracentrifuge[3] and paper electrophoresis,[4] and the recent reports of Volwiler's group[5] on the comparison of apparent turnover times of several $S^{35}$ labeled fractions synthesized in vivo following oral ingestion of the isotope and intravenously injected following in vitro plasma fractionation, all emphasize a problem which Volwiler and associates[5] have phrased thus: "Iodinization of plasma proteins may sufficiently denature protein as to result in a shortened life span."

Nevertheless, the $I^{131}$ label, by reason of availability and convenience, is achieving increased usage in studies aimed at elucidation of synthesis and breakdown rates of plasma protein fractions; gamma globulins[6, 7] and the beta metal-combining globulin[8] are the subject of very recent communications. Preparations of $I^{131}$ labeled gamma globulins are now commercially available to investigators.†

From the Hektoen Institute of Medical Research of the Cook County Hospital, and the Department of Medicine of the University of Illinois College of Medicine.

Received for publication Aug. 7, 1954.

*The senior author is indebted to the Dr. Leonard H. and Louis D. Weissman Medical Research Foundation for the backing of the studies of plasma proteins as related to disease mechanisms at the Hektoen Institute for Medical Research of the Cook County Hospital.

**Undergraduate Research Fellow in Medicine under a grant from the United States Public Health Service. Some of the work of this paper was submitted in a thesis in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry at the University of Illinois College of Medicine.

***Undergraduate Research Fellow in Medicine under a grant from the United States Public Health Service.

****University of Illinois Research Fellow in Medicine at the initiation of this work. Presently at the Research and Educational Hospitals of the University of Illinois.

†Abbott Laboratories, North Chicago, Ill.

Thus it has seemed of interest to pursue our comparison of apparent turnover times following $S^{35}$ feeding and following intravenous injection of the $I^{131}$ I), the $S^{35}$ apparent turnover times of A. W. (42 days) and P. E. (47 days)

## CHOICE OF SUBJECTS AND PLAN OF STUDY

The two subjects, P. E. and A. W., 52-year-old men, were selected less by reason of normality than by reason of availability to cooperate in long-term metabolic studies in which, in successive periods, each might serve as "his own control." Both have been long-term residents of the neurological ward at Cook County Hospital, P. E. because of moderately advanced Huntington's chorea, A. W. because of a local stabilized lesion in the right anterior quadrant of the cervical spinal cord, to which (though believed to be on a vascular basis) no pathologic diagnosis can be ascribed with certainty at this time.

Both subjects were in good nutritional status; neither gave evidence of metabolic, renal, or hepatic disease by usual clinical criteria. The Bromsulphthalein test (5 mg. per kilogram) showed no abnormal retention in either subject.

The distribution of electrophoretic plasma-protein component nevertheless differed significantly and consistently between the two. Representative data during the $S^{35}$ study period are given in Table I, and show agreement between free and paper electrophoresis within the limits expected both from our previous studies with identical techniques[1] and from the studies of Mackay and co-workers[9] whose paper estimates are photometric rather than gravimetric. (See also the studies of Wurm.[10])

TABLE I. COMPARISON OF DISTRIBUTION OF COMPONENTS* BY FREE AND PAPER ELECTROPHORESIS AT ABOUT THREE AND SIX WEEKS AFTER $S^{35}$ INGESTION

| SUBJECT | TIME (WK.) | METHOD | RELATIVE CONCENTRATION OF FRACTION (%) | | | | |
|---|---|---|---|---|---|---|---|
| | | | ALBUMINS | $\alpha_1$ | $\alpha_2$ | $\beta$ | $\gamma$ |
| P. E. | 3 | Free | 53 | 4 | 12 | 18 | 13 |
| | | Paper | 55 | | 29 | | 16 |
| | 6 | Free | 54 | 4 | 12 | 15 | 15 |
| | | Paper | 49 | | 31 | | 18 |
| A. W. | 3 | Free | 46 | 5 | 13 | 13 | 23 |
| | | Paper | 42 | | 35 | | 23 |
| | 6 | Free | 43 | 6 | 11 | 15 | 25 |
| | | Paper | 38 | | 35 | | 27 |

*Fibrinogen was gravimetrically determined. For subject P. E. it averaged 290 mg. per cent; for subject A. W., 450 mg. per cent.

Whereas the elevated gamma globulin and fibrinogen levels in A. W. may prove of significance in the ultimate appraisal of the etiology of his lesion, for this study the significance lies solely in permitting comparison of apparent turnover times of these gross electrophoretic components with those of P. E., whose levels were not observed outside of normal limits.[11]

Each subject was fed 0.5 mc. $S^{35}$ labeled yeast;* thereafter blood samples were withdrawn at approximate weekly intervals for seven weeks. During the $S^{35}$ experimental periods subjects were given daily oral supplements of 2 Gm. cystine and 4 Gm. methionine. (Multiple blood samples in the first week were not taken, because in our hands analysis of the radioactivity levels during the "equilibration" phase has not permitted reliable resolution in terms of more than one rate constant, and thus is not likely to throw light on the question of multiple extravascular "compartments" raised by Berson and Yalow.[12])

The use of a seven-week period of observation for the "metabolic" phase of albumins and gamma globulins represents an effort to seek indication of slower rates of biologic

*Abbott Laboratories, North Chicago, Ill.

turnover after the two- to three-week period usually employed for characterization of protein "biologic half lives."

About eight weeks after the end of the $S^{35}$ period each subject received 5 mg. gamma globulin* labeled with $I^{131}$ (representing radiation dosage of 60 microcuries) intravenously.† During the ensuing $I^{131}$ study period subjects received Lugol's solution, 30 drops a day, by mouth, and although radioactivity by the end of five weeks was too low for interpretation, blood was drawn for fractionation and radioactivity measurements at approximately weekly intervals for seven weeks by the techniques and for the reasons given in the next section.

## FRACTIONS STUDIED AND METHODOLOGICAL CONSIDERATIONS

Three crude fractions, namely albumins, a mixture of alpha$_1$ alpha$_2$ beta globulins, and gamma globulins separated from serum by paper electrophoresis, are the basis of this study. After $I^{131}$ gamma-globulin intravenous injection, the techniques of separation of fractions, film preparation, and radioactivity determinations have been identical with those detailed in our previous report.[1]

In addition, in both subjects we have made estimates of the apparent turnover time of fibrinogen as follows (based on the practices of Morrison[13] and Madden and Gould[14]):

To approximately 10 ml. of oxalated plasma is added three times its measure volume of 0.15 M NaCl in a Petri dish. A flat clot is made by adding 10 N.I.H. units bovine thrombin‡ for each 2.5 ml. of plasma. Clotting is allowed to proceed for at least 8 hours at 5° C.

These clots undergo syneresis readily.[15] The clot is transferred to a flat surface of several layers of filter paper; most of the solute phase is expressed by pressure and kneading with a spatula; the lump of wet fibrin is quantitatively transferred to a test tube and washed with kneading three times with about 20 ml. 0.15 M NaCl and three times with 20 ml. distilled water.

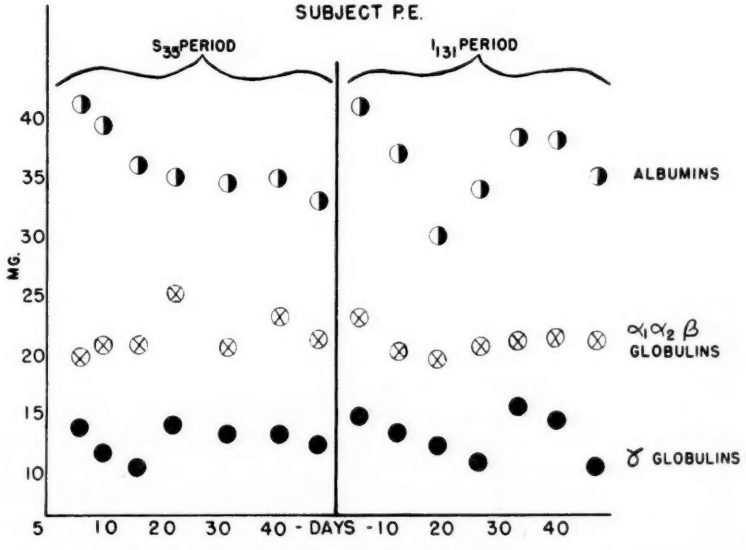



Fig. 1.—For description, see text.

The clot is then dissolved by partial hydrolysis in approximately 5 ml. 6 N HCl over a steam bath for six hours, with frequent agitation. The hydrolysate is then transferred to a watch glass, taken up in 3 ml. distilled water and re-evaporated to dryness three times. The hydrolysate is then redissolved in a minimum amount of distilled water, transferred

*Abbott Laboratories, North Chicago, Ill., Control number 806-151-2.

†In subject P. E. an estimated 10 per cent of the dose was accidentally injected subcutaneously.

‡Parke, Davis & Company, Detroit, Mich.

quantitatively to a tared steel counting cup, and evaporated to dryness at 110° C.; and the dry weight recorded. The film is stored for counting in a desiccator over solid NaOH.

It has been our practice to prepare duplicate cloths for each bleeding.

*Reproducibility of Weight of Extracted Fractions.*—We have already stressed the necessity for technical meticulousness in this step to achieve reproducibility. This necessity is again emphasized by the data illustrated in Fig. 1. Here are plotted, as they lie sequentially in raw data books, weights of electrophoretically separated fractions of subject P. E. during the $S^{35}$ and $I^{131}$ study periods. Errors in initial pipetting, in extraction, in precipitation of protein and washing, all are cumulative supplements to the inherent crudeness of paper electrophoretic separation to give this scatter of values.

The scatter is somewhat greater than has been observed in our laboratory when a single worker has been responsible for these steps[1]; in this study more than one worker has carried out extractions. Therefore it has been necessary at frequent intervals to obtain duplicate and triplicate repetitions of the procedure underlying each experimental point in subsequent figures in order to convince ourselves that levels of the components were not undergoing significant change, and to give validity to the type of data presented in Table I of this paper and Table II of its predecessor.[1] For this reason, also, in the $I^{131}$ period separations were made and weights of fractions measured for two weeks after radioactivity had fallen below measurable limits.

TABLE II. WEIGHTS OF COUNTED FRACTIONS AT BEGINNING AND END OF EXPERIMENTAL PERIODS

| | FIBRINOGEN* (MG. %) | | ALBUMINS† (GM. %) | | $\alpha_1\ \alpha_2\ \beta$† (GM. %) | | $\gamma$† (GM. %) | |
|---|---|---|---|---|---|---|---|---|
| SUBJECT | BEGINNING | END | BEGINNING | END | BEGINNING | END | BEGINNING | END |
| P. E. | 300 | 280 | 4.3 | 3.8 | 2.0 | 2.1 | 1.4 | 1.6 |
| A. W. | 470 | 440 | 3.5 | 3.3 | 2.9 | 2.1 | 2.1 | 2.0 |

*Interval: Approximately seven weeks.
†Interval: Approximately five months.

For, as usually estimated from "die away" semilogarithmic isotopic plots, the apparent turnover time implies constant and equal rates of synthesis and degradation during the experimental period. An increase in synthesis without comparable simultaneous increase in degradation will lead to steepening of slope and consequently a temporary shortening of the "time" by reason of progressive dilution of the isotopically labeled protein. A temporary increase in degradation without corresponding increase in synthesis could be graphically indistinguishable in terms of isotopic data alone. Differentiation is possible only in terms of a change in the total amount of component under study, which may or may not be reflected by a change in its concentration in plasma.

We have made no attempt to estimate the size of the exchangeable albumin and gamma-globulin pools by $I^{131}$ labeled protein-dilution techniques at the beginning and end of the study periods: first, because such studies might increase the length of the experimental period to a point where the chance of failure of the whole experiment by reason of intercurrent infection or through temperamental upheaval, and "signing out" becomes large; second, because no direct comparisons of $I^{131}$ values with values estimated by $S^{35}$ are possible when the $S^{35}$ is administered orally.[1]

Therefore we have contended ourselves with constant scrutiny of changes of concentrations of components as estimated not merely from free electrophoresis (e.g., Table I), but from the recorded weights of the salt-free protein films subjected to radioactive measurements.

In Table III are recorded such weights at the beginning and end of the *total* ($S^{35}$ + interval + $I^{131}$ period) for the electrophoretically separated fractions, and for fibrinogen (the beginning and end of the $S^{35}$: period). The only difference that we believe may lie beyond the limits of week-to-week reproducibility in multiple experimental hands is that between the $alpha_1$ $alpha_2$ beta fraction in subject A.W. No clinical events correspond to this apparent shift.

*Quality of Films for Counting and Reproducibility of Radioactive Determinations.*—We have graded our films, prepared as previously described,[1] thus:

*Grade I: Excellent.* No curling, cracking, or unevenness. Film paper lies flat in counting cup.

*Grade II: Good.* Either minimal cracking or curling but with no other unevenness. Paper lies flat in counting cup.

*Grade III: Fair.* Both minimal curling and cracking. No unevenness in center. Paper lies flat in counting cup.

*Grade IV: Poor.* Minimal to moderate cracking, curling, and unevenness. Paper lies flat in planchet.

*Grade V: Discarded.* Marked cracking, curling, and unevenness throughout film. Paper does not lie flat in planchet. This is a rejected film and is discarded from the data.

TABLE III. SELF-ABSORPTION CORRECTION FACTORS FOR $I^{131}$ LABELED DRIED PLASMA PROTEIN FILMS*

| WEIGHT OF FILM (MG.) | F | WEIGHT OF FILM (MG.) | F |
|---|---|---|---|
| 3.0 | 12.0 | 10.0 | 2.8 |
| 3.1 | 11.8 | 10.5 | 2.7 |
| 3.2 | 11.6 | | |
| 3.3 | 11.4 | 11.0 | 2.6 |
| 3.4 | 11.2 | 11.5 | 2.5 |
| 3.5 | 11.0 | | |
| 3.6 | 10.8 | 12.0 | 2.4 |
| 3.7 | 10.6 | 12.5 | 2.3 |
| 3.8 | 10.4 | | |
| 3.9 | 10.2 | 13.0 | 2.2 |
| | | 13.5 | 2.1 |
| 4.0 | 9.9 | | |
| 4.1 | 9.2 | 14.0 | 2.0 |
| 4.2 | 8.6 | 14.5 | 1.95 |
| 4.3 | 8.4 | | |
| 4.4 | 8.0 | 15.0 | 1.9 |
| 4.5 | 7.8 | 16.0 | 1.8 |
| 4.6 | 7.6 | 17.0 | 1.7 |
| 4.8 | 7.4 | 18.0 | 1.65 |
| 4.9 | 7.2 | 19.0 | 1.58 |
| 5.0 | 6.8 | 20.0 | 1.52 |
| 5.1 | 6.2 | 21.0 | 1.44 |
| 5.2 | 5.8 | 22.0 | 1.40 |
| 5.3 | 5.7 | 23.0 | 1.38 |
| 5.4 | 5.6 | 24.0 | 1.36 |
| 5.5 | 5.5 | 25.0 | 1.33 |
| 5.6 | 5.4 | 26.0 | 1.31 |
| 5.7 | 5.3 | 27.0 | 1.30 |
| 5.8 | 5.2 | 28.0 | 1.28 |
| | | 29.0 | 1.25 |
| 6.0 | 5.0 | | |
| 6.2 | 4.7 | 30.0 | 1.22 |
| 6.4 | 4.6 | 31.0 | 1.20 |
| 6.6 | 4.5 | 32.0 | 1.19 |
| 6.8 | 4.2 | 33.0 | 1.18 |
| | | 34.0 | 1.17 |
| 7.0 | 4.0 | 35.0 | 1.16 |
| 7.2 | 3.8 | 36.0 | 1.15 |
| 7.6 | 3.7 | 37.0 | 1.14 |
| 7.8 | 3.6 | 38.0 | 1.13 |
| | | 39.0 | 1.12 |
| 8.0 | 3.5 | | |
| 8.5 | 3.3 | 40.0 | 1.11 |
| | | 45.0 | 1.05 |
| 9.0 | 3.1 | | |
| 9.5 | 2.95 | 50.0 | 1.02 |
| | | 60.0 | 1.00 |

*Average film diameter is 1.80 cm. To express the above table in milligrams per square centimeter, each figure in the first column may be divided by 2.53.

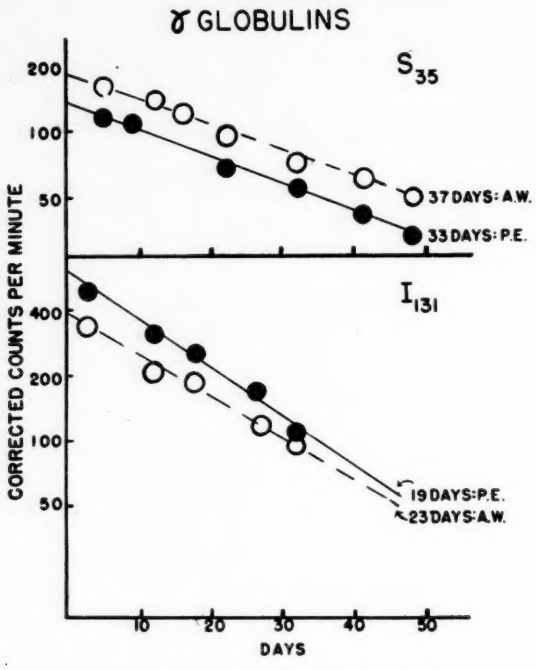



Fig. 2.—For description, see text.

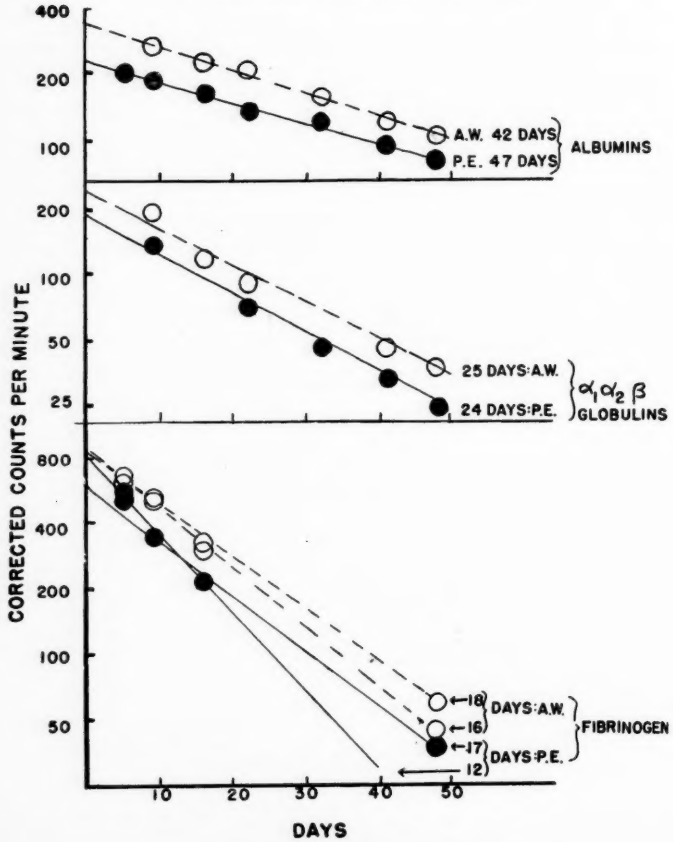



Fig. 3.—For description, see text.

Eighty-five per cent of the prepared films fell between Grades I and III; the discarding of films in Grades IV and V is evident from absence of experimental points at regular weekly intervals in Figs. 2 and 3.

Reproducibility of counting for acceptable films (the per cent difference from the mean of the duplicate count) was less than 5 per cent in all measurements and less than 1 per cent in over 50 per cent of them.*

*Layer (Self-absorption) Correction of $I_{131}$ Radioactivity Determinations.*—Because the range of weights of gamma globulins extracted from the sera of the two subjects of this study fall in a section of the layer correction curve (Fig. 4, *A* in the previous paper[1]) where graphical interpolation is difficult, the data on which this curve is based are given in Table III.

## RESULTS

By the custom of our laboratory we express results in terms "apparent turnover times" in preference to "biologic half lives" to avoid any implication that the values obtained from graphical analysis of semilogarithmic radioactivity plots of fractions as crude as are obtained by electrophoresis can characterize the biologic behavior of any single protein molecular species. (For comparison with data in the literature: biologic half life = apparent turnover time × log 2.)

The plots of Figs. 2 and 3 are semilogarithmic; the ordinates are radioactivity corrected for physical decay and self-absorption; the abscissas are time in days.

*Albumins.*—Despite significant differences in serum albumin levels (Table I), the $S^{35}$ apparent turnover times of A.W. (42 days) and P.E. (47 days) differed by little more than the expected error of graphical analysis (Fig. 2, top). Moreover, a glance at the plot gives no evidence of a disappearance rate significantly different at the end from that at the beginning of the seven-week study period.

The $S^{35}$ values for both subjects are significantly longer than any as yet obtained with intravenous $I^{131}$ labeled preparations in the hands of others or ourselves (see Table I[1]). They are of a comparable order of magnitude (though not quite as long) as certain of the values we have reported following $S^{35}$ ingestion in subjects with no evident metabolic disease, and are in striking agreement with the value of 42 days (29 days/log 2) reported by Volwiler and co-workers[5] for normal human albumins fractionated by Method 10[16, 17] following $S^{35}$ ingestion.

During the $I^{131}$ experimental period, the radioactivity measurable in the albumin fractions lay within the range directly predictable from the biologic decay curves for the $S^{35}$ period of Fig. 2. Thus, there we found no evidence of a reversed "tailing,"[1] i.e., of $I^{131}$ radioactivity injected with the gamma globulins appearing in the albumin fractions.

*Globulins of Intermediate Mobility.*—The apparent turnover times of this very gross mixture of $alpha_1$ $alpha_2$ beta globulins in both subjects (Fig. 2, middle) were slightly over half the corresponding albumin figures, a result consistent with our previously reported findings. Shorter apparent turnover times have been recently indicated for purer components (e.g., beta metal-bind-

*Counting was carried out, both for $S^{35}$ and $I^{131}$ periods, in a windowless gas flow counter (Tracerlab SC16).

ing globulin and beta lipoprotein). It is probable that the influence of these components is overbalanced by slower turnover components in the mixture. A suggestion of this is given by the position of experimental points at the end of ten days *above* the lines drawn through the points of the final four weeks (Fig. 2). This is not seen in either the albumins or gamma-globulins plots (Fig. 3).

During the $I^{131}$ period a small and inconstant amount of the total radioactivity was extracted in the fraction of intermediate mobility. Higher than that predictable from biologic decay in the $S^{35}$ period, this amount doubtless represents incomplete segregation of the injected labeled gamma globulins in the gamma position on the paper.

*Fibrinogen.*—The data for fibrinogen (Fig. 2, bottom) indicate apparent turnover times considerably shorter than those of the albumins but somewhat longer than the range reported for normal man following $S^{35}$ ingestion by Madden and Gould.[14] Because the experimental points do not fall on a straight line in the semilogarithmic plot, we have indicated the extremes of range of apparent turnover times they suggest in preference to arbitrary assignment of single values for each subject. The problems raised by these findings are presently under investigation and will be the subject of a subsequent communication.

*Gamma Globulins.*—In Fig. 3 (top) are given the data following $S^{35}$ ingestion. They lead to apparent turnover times (37 days and 33 days) differing by little more than expected from the experimental error of the methods, and of the order of magnitude consistent with Volwiler and co-workers' value (39 days) for the normal.

In Fig. 3, bottom, are plotted the data following $I^{131}$ gamma-globulin intravenous injection. The clearly steeper slopes, as contrasted to the lines of Fig. 3, top, correspond to an apparent turnover time for each subject only about 60 per cent of that derived from the $S^{35}$ data.

The mean of these values (21 days) agrees with the mean of Eisenmenger and Slater's[7] values (22 days) and Havens and co-workers'[19] average value (20 days). All these are somewhat longer than the 10 days derived from the report of Vaughan and associates.[6]

## DISCUSSION

These discrepancies in apparent turnover times between $I^{131}$ and $S^{35}$ labeled gamma globulins found in both subjects closely resemble, in magnitude and implications, our previously reported discrepancies between apparent turnover times of albumins when measured with these two labeling techniques in the same individual.

It is true that these discrepancies reinforce the suspicion stated in our introduction: Iodination of a protein may result in a shortened in vivo life span. But they do not prove this suspicion.

For it is not unthinkable that the peptide bonds around $I_{131}$ bearing amino acids in a protein have differing rates of opening and closing than those around $S^{35}$ bearing amino acid; that is to say, the synthesis and degradation of a protein molecule consisting of hundreds of amino acid residues is not an "all or none" phenomenon.

Moreover, it is by no means proved that the longer apparent turnover times following $S^{35}$ feeding are uninfluenced by some continuous resynthesis of protein from $S^{35}$ released in degradation during the "die away" period.

It is true that Volwiler and co-workers[5] recently injected plasma from subjects who have received $S^{35}$ orally into subjects who have not, and has reported comparable apparent turnover times for the fractions studied in the two groups. This report goes a long way to dispel the suspicion of "reutilization." In our opinion the suspicion cannot be dispelled completely until similar findings emerge from comparable studies in disease states where variations may exist in the type and magnitude of S-metabolic pools involved in plasma-protein synthesis and degradation.

Again, the feeding of oral supplements of unlabeled S containing amino acids to minimize protein resynthesis from the biologically degraded isotope may well be groundless if the resynthesis occurs from moieties larger than single amino acids. Thus such feeding cannot a priori rule out a contribution of resynthesis to the measured apparent turnover times.

We therefore believe that only very large differences from the "normal" values for apparent turnover times can be given metabolic significance in the analysis of disease sequences in the existing state of our knowledge. We feel further comparative studies using different isotopic labels in the same subjects are necessary properly to evaluate the nature of existing discrepancies.

The closely comparable apparent turnover times for all fractions here studied, despite sustained differences in blood levels, reinforce the independence of life span and circulating mass suggested in the earlier work of this laboratory and other laboratories.[1, 5]

## SUMMARY

In two* subjects, following ingestion of $S^{35}$ labeled yeast, we have determined the apparent turnover times of the following four plasma protein fractions: albumins, a mixture of $alpha_1$ $alpha_2$ globulins, gamma globulins (separated by paper electrophoresis), and fibrinogen.

Despite consistently differing blood levels of these fractions, both subjects showed closely comparable apparent turnover times. For albumins these were 42 and 47 days; for globulins of intermediate mobility, 25 and 24 days; for gamma globulins, 33 and 37 days, respectively.

In a second experimental period the apparent turnover time of the gamma globulins following intravenous injection of an $I^{131}$ labeled preparation was determined in the same subjects. The values were 19 days and 23 days, respectively; or approximately 60 per cent of the comparable $S^{35}$ values.

The implications of these findings are discussed from standpoints of methodology and of metabolic interpretation of variations of "normal" of apparent turnover times in disease states.

*While this paper has been in press, we have completed a similar study on a third subject and have found a comparable discrepancy between the apparent turnover time of the gamma globulins as measured following ingestion of $S^{35}$ and following injection of $I^{131}$ labeled gamma globulins. In contrast to the two subjects reported here, our third subject gave evidence of an even slower apparent turnover time measured from the slope of the $S^{35}$ plot subsequent to the initial fortnight following injection than that estimated from the slope of the plot during the initial fortnight.

We are grateful to the Otho S. A. Sprague Memorial Institute for funds to initiate this series of studies; to Miss A. Gomoll for technical assistance; and to Miss J. Peters and Miss R. Quarum of the Neurological Service at Cook County Hospital for metabolic nursing assistance.

## REFERENCES

1. Armstrong, S. H., Jr., McLeod, K., Wolter, J., and Kukral, J.: The Persistence in the Blood of the Radioactive Label of Albumin, Gamma Globulins, Globulins of Intermediate Mobility Studied With $S_{35}$ and Paper Electrophoresis: Methods and Preliminary Results, J. LAB. & CLIN. MED. **43**: 918, 1954.
2. London, I. M.: Studies on the Rates of Formation of Serum Proteins in Man *in* Symposia on Nutrition. II. Plasma Proteins, edited by J. B. Youmans, Springfield, Ill., 1949, Charles C Thomas, Publisher.
3. Berson, S. A., Yalow, R. S., Schreiber, S. S., and Post, J.: Tracer Experiments with $I_{131}$ Labeled Human Serum Albumin: Distribution and Degradation Studies, J. Clin. Invest. **32**: 746, 1953.
4. Gabrieli, E. R., Goulian, D., Jr., Kinersly, T., and Collet, R.: Zone Paper Electrophoresis Studies on Radio-Iodinated Human Serum Albumin, J. Clin. Invest. **33**: 136, 1954.
5. Volwiler, W., Goldsworthy, P. D., MacMartin, M. P., Wood, P. A., Mackay, I. R., Fremont-Smith, K., and Shook, D. F.: Turn-over Rates of Various Plasma Proteins in Normal and Cirrhotic Humans Using Biosynthetic Methods, J. Clin. Invest. **33**: 970, 1954.
6. Vaughan, J. H., Armato, J., Goldwater, P., Brachman, C., Favour, B., and Bayles, T. B.: Fate of Intravenously Injected Labeled Gamma Globulin in the Normal Subject and in Patients with Rheumatoid Arthritis, J. Clin. Invest. **31**: 668, 1952.
7. Eisenmenger, W. J., and Slater, R. J.: Distribution and Decay of $I_{131}$ Tagged Albumin and Gamma Globulin in Patients with Cirrhosis, J. Clin. Invest. **32**: 564, 1953.
8. Elmlinger, P. J., Masouredis, S. P., Soni, J., Fulton, G. E., and Belknap, S. L.: Plasma Mixing and Removal Curves for $Fe_{59}$ and $I_{131}$ after Intravenous Injection of Doubly Labeled Iron Binding Globulin, J. Clin. Invest. **33**: 930, 1954.
9. Mackay, I. R., Volwiler, W., Goldsworthy, P. D., Eriksen, N., and Wood, P. A.: Paper Electrophoresis of Serum Proteins: Photometric Quantitation and Comparison with Free Electrophoresis, J. Clin. Invest. **33**: 855, 1954.
10. Wurm, M.: Quantitative Fractionation of Serum Proteins Determined by Moving Boundary and Paper Electrophoresis, J. Clin. Invest. **33**: 973, 1954.
11. Armstrong, S. H., Jr., Budka, M. J. E., Morrison, K. C., and Hasson, M.: Preparation and Properties of Serum and Plasma Proteins. XII. The Refractive Properties of the Proteins of Human Plasma and Certain Purified Fractions, J. Am. Chem. Soc. **69**: 1747, 1947.
12. Berson, S. A., and Yalow, R. S.: The Distribution of $I_{131}$ Labeled Human Serum Albumin Introduced into Ascitic Fluid: Analysis of the Kinetics of a Three Compartment Catenary Transfer System in Man and Speculations on Possible Sites of Degradation, J. Clin. Invest. **33**: 377, 1954.
13. Morrison, P. R.: Preparation and Properties of Serum and Plasma Proteins. XV. Some Factors Influencing the Quantitative Determination of Fibrinogen, J. Am. Chem. Soc. **69**: 2723, 1947.
14. Madden, R. E., and Gould, R. G.: The Turnover Rate of Fibrinogen in the Dog, J. Biol. Chem. **196**: 641, 1952.
15. Ferry, J. D. and Morrison, P. R.: The Conversion of Human Fibrinogen to Fibrin under Various Conditions, J. Am. Chem. Soc. **69**: 388, 1947.
16. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K., and Uroma, E.: A System for the Separation of the Components of Human Blood: Quantitative Procedures for the Separation of the Protein Components of Human Plasma, J. Am. Chem. Soc. **72**: 465, 1950.
17. Lever, W. E., Gurd, F. R. N., Uroma, E., Brown, R. K., Barnes, B. A., Schmid, K., and Schultz, E. L.: Chemical, Clinical and Immunological Studies on the Products of Human Plasma Fractionation. XL. Quantitative Separation and Determination of the Protein Components in Small Amounts of Normal Human Plasma, J. Clin. Invest. **30**: 99, 1951.
18. Volwiler, W., Fremont-Smith, K., and MacMartin, M. P.: Turnover Rates of Serum Proteins in Cirrhotic Man, J. Clin. Invest. **31**: 668, 1952.
19. Havens, W. P., Jr., Dickensheets, J., Bierly, J. N., and Eberhard, T. P.: The Half-Life of Normal Human Gamma Globulin in Patients with Hepatic Cirrhosis, J. Clin. Invest. **32**: 573, 1953.

# CHANGES IN PROTEIN FRACTIONS OF DOG PLASMA AFTER BLEEDING AND DEXTRAN INFUSION

ROBERT E. SEMPLE, M.A., PH.D.
KINGSTON, ONTARIO

## INTRODUCTION

INFUSIONS of dextran under a wide range of experimental and clinical conditions result either in no appreciable change[1, 2] or in minor decreases[3, 4, 5] in total circulating protein. Decreases reported were small and have not been considered of practical importance.[3] On the other hand, there is evidence that dextran results in a change in the composition of the circulating protein, but there have been differences of opinion in regard to the nature of such a change. Thus Thorsén[3] has reported that the albumin fraction is decreased to a greater extent than the globulin, whereas Hetzel[6] has observed a postinfusion increase in the albumin-globulin ratio and concludes that the globulin fraction is preferentially decreased as a result of dextran infusion. Few reports are available of effects on fibrinogen. Small infusions in normal dogs[2] failed to produce any appreciable change in total fibrinogen, but dextran and fibrinogen have been reported to combine, in vitro, to produce a precipitate.[7] In addition there have been reports of dextran acting to prolong wound bleeding.[5, 8] The present study will show that the only measurable effect of dextran infusions on the albumin fraction is dilution, but that there is a decrease in fibrinogen concentration greater than can be accounted for by plasma dilution.

The possibility of infused colloid inhibiting the mobilization or regeneration of endogenous proteins has been suggested.[9, 10] The evidence to be presented, however, indicates that after large dextran infusions the return of proteins to control concentrations proceeds at the same rate and follows the same pattern as that observed when no exogenous colloid is present.

## METHODS

Ten adult mongrel dogs were bled through a cannulated femoral artery, and the lost blood, which amounted to about 45 per cent of control blood volume, was replaced by a 5.7 per cent solution of dextran* in 0.9 per cent saline. The details of the bleeding and infusion procedures have been described.[5, 11]

Blood samples were obtained from an external jugular vein at times indicated by Table I and Fig. 1, and were placed in tubes containing sufficient potassium oxalate to make up a 0.2 per cent solution of the oxalate in whole blood. Samples were centrifuged and the resulting oxalated plasma was used for the protein determinations. Measurements were made of total nitrogen and the nitrogen remaining after complete protein removal with trichloroacetic acid. Globulins were precipitated with 23 per cent sodium sulfate[12]

From the Department of Physiology, Faculty of Medicine, Queen's University, Kingston, Ontario.

Supported by Medical Grant No. 251 from the Defence Research Board of Canada.
Received for publication June 28, 1954.

*Some characteristics of this dextran preparation, an investigational product of the Connaught Medical Research Laboratories, Toronto, Canada, have been described.[2]

and the aqueous filtrate[13] analyzed for albumin nitrogen. The method of Cullen and Van Slyke[14] was used to measure fibrinogen. Preliminary tests indicated that the addition of 2 to 4 National Institutes of Health units of thrombin* to the calcium chloride solution used to precipitate fibrinogen ensured rapid and apparently complete recovery from both plasma with normal fibrinogen concentration and plasma with a fibrinogen concentration reduced to one-third by dilution with 0.9 per cent saline. Thus in four such tests recovery from diluted samples averaged 98.8 per cent of that from undiluted plasma (range 97.0 to 103.1 per cent). In all protein determinations the nitrogen, in the form of the ammonium radical, was measured by titration after micro-Kjeldahl digestion and distillation. Appropriate corrections were made for nonprotein nitrogen and the nitrogen concentrations so determined were multiplied by 6.25 to give the protein concentration of the plasma.

In six experiments T-1824 was used to measure plasma volumes during control periods, after bleeding, and immediately after dextran infusions.

TABLE I

| EXPERIMENT | GRAMS PROTEIN PER 100 ML. PLASMA | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CONTROL | | | 45 TO 60 MINUTES AFTER BLEEDING, JUST BEFORE DEXTRAN INFUSION | | | TIME AFTER DEXTRAN INFUSION | | | | | |
| | | | | | | | 5 TO 15 MINUTES | | | ONE HOUR | | |
| | A | G | F | A | G | F | A | G | F | A | G | F |
| 1 | 3.82 | 1.21 | 0.230 | 3.17 | 1.14 | 0.195 | 1.48 | 0.58 | 0.066 | 1.46 | 0.60 | 0.072 |
| 2 | 3.67 | 1.75 | 0.223 | 3.16 | 1.40 | 0.170 | 1.17 | 0.64 | 0.062 | 1.26 | 0.60 | 0.062 |
| 3 | 3.08 | 1.83 | 0.196 | 2.71 | 1.51 | 0.165 | 1.04 | 0.46 | 0.051 | 1.22 | 0.38 | 0.044 |
| 4 | 3.89 | 2.12 | 0.227 | 3.12 | 1.57 | 0.170 | 1.39 | 0.60 | 0.036 | 1.41 | 0.80 | 0.039 |
| 5 | 3.19 | 2.98 | 0.381 | 2.85 | 2.53 | 0.309 | 1.26 | 0.96 | 0.141 | 1.27 | 1.00 | 0.161 |
| 6 | 2.18 | 2.70 | 0.204 | 1.81 | 2.41 | 0.169 | 1.23 | 0.81 | 0.065 | 1.30 | 0.84 | 0.070 |
| 7 | 3.07 | 1.09 | 0.231 | 2.57 | 1.25 | 0.193 | 1.19 | 0.60 | 0.073 | 1.23 | 0.56 | 0.078 |
| 8 | 3.21 | 2.17 | 0.239 | 2.76 | 2.16 | 0.218 | 1.33 | 0.65 | 0.082 | 1.23 | 0.86 | 0.087 |
| 9 | 3.16 | 2.49 | 0.251 | 2.91 | 2.26 | 0.231 | 1.52 | 1.36 | 0.104 | 1.42 | 1.13 | 0.116 |
| 10 | 3.37 | 2.19 | 0.268 | 3.05 | 2.05 | 0.249 | 1.66 | 0.54 | 0.110 | 1.62 | 0.77 | 0.116 |
| Means | 3.26 | 2.05 | 0.245 | 2.81 | 1.83 | 0.207 | 1.33 | 0.72 | 0.079 | 1.34 | 0.75 | 0.085 |
| Per cent preinfusion | | | | | | | 47.2 | 39.3 | 38.2 | 47.8 | 41.0 | 41.1 |

Albumin (A), globulin (G), and fibrinogen (F) concentrations during control periods, after bleeding, and after dextran infusions.

## RESULTS

Changes in plasma protein concentration as a result of bleeding and those recorded immediately and one hour after infusion are reported in Table I. Subsequent observations are summarized by Fig. 1.

Bleeding resulted in a 11 to 16 per cent mean dilution of each of the measured fractions by protein-poor interstitial fluid. Dextran infusions reduced the albumin fraction to 47.2 per cent of the mean preinfusion concentration. The mean globulin concentration was reduced to 39.3 per cent and fibrinogen to 38.2 per cent of preinfusion levels. The decrease in globulins was not significantly greater than was the decrease in albumin, but the fibrinogen fraction was lowered to a significantly greater extent than was the albumin ($P = 0.016$). The concentrations observed in the various fractions one hour after infusion were somewhat greater than those noted 5 to 15 minutes after infusion, with the fibrinogen fraction showing the largest relative

*Thrombin Topical (bovine — Parke, Davis & Company, Ltd., Walkerville, Ontario).

increase. Fig. 1 shows that there followed significant increases in the concentrations of all fractions. Fibrinogen increased rapidly and the concentration was within control range in 24 hours. Albumin concentrations were still significantly lower than the control 72 hours after infusion but at 96 hours were not significantly different from controls. The globulins were restored to control concentrations at about the same rate as was the albumin (Fig. 1).

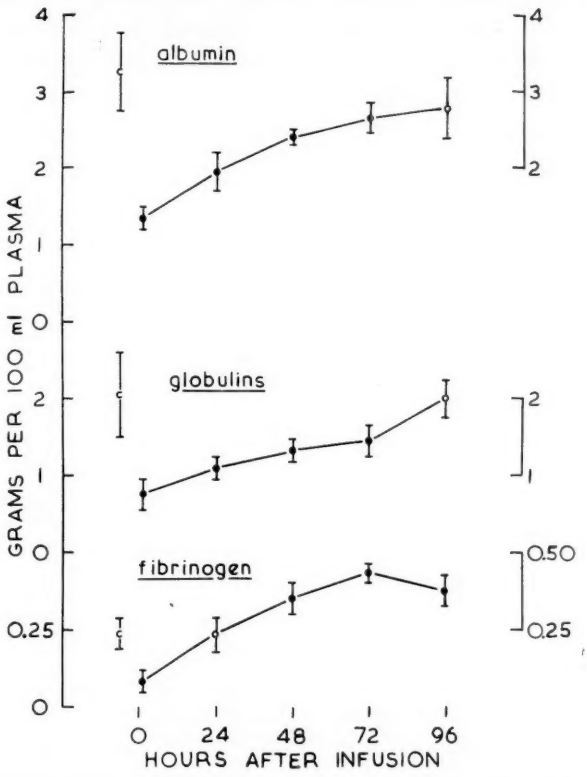



Fig. 1.—Restoration of protein fractions after bleeding followed by dextran infusion. End of infusion taken as zero time. Dots locate means that are significantly different from appropriate controls ($P = 0.05$ or less); means marked by circles are not significantly different from controls. Control means (c) are shown to left of zero time. Vertical lines locate one S.D. on either side of each mean.

Plasma volume changes were essentially the same as those reported earlier for a larger series of similarly treated dogs.[5]

## DISCUSSION

The amount of protein in the circulating plasma before and after dextran infusions should not be appreciably different provided there is no appreciable mobilization of protein during infusion and if the infused colloid acts only to dilute the protein present. Calculated data presented in Table II show that, within the limits of experimental error, the albumin fraction is thus diluted. Assuming albumin balance, fibrinogen concentrations observed after infusions represent a loss of fibrinogen that varied with the animal under ob-

servation, but averaged about 20 per cent of the fibrinogen present before infusion. Ricketts[7] has shown that in vitro mixing of fibrinogen and dextran solutions results in the precipitation of a fibrinogen-dextran complex that includes from 9 to 13 per cent of the total fibrinogen present. The loss of fibrinogen noted in the present series of experiments may be the result of an in vivo reaction similar to the in vitro combination described by Ricketts. Although small and possibly of little clinical importance, the loss of fibrinogen that results from dextran infusions is of interest. It may be partly responsible for the excess wound bleeding sometimes noted after dextran administration. The globulin fraction was measured "by difference" and thus the experimental error in this estimation is relatively large. Dilution alone could account for the decreases observed in globulin concentration but the evidence is not conclusive.

TABLE II

| EXPERIMENT | GRAMS ALBUMIN IN PLASMA | |
|---|---|---|
| | BEFORE INFUSION | AFTER INFUSION |
| 1 | 6.85 | 6.66 |
| 2 | 8.37 | 7.75 |
| 3 | 6.01 | 6.51 |
| 4 | 6.99 | 7.01 |
| 8 | 12.56 | 12.98 |
| 9 | 15.27 | 15.40 |
| Means | 9.34 | 9.39 |

Total circulating albumin (plasma volume × albumin concentration) in six dogs before and after dextran infusions.

In order to determine whether or not the dextran present in the plasma interferes with the return of the protein fractions to normal concentrations, it is necessary to compare the results noted above with a study in which no foreign colloid was used. Zeldis and Alling[15] have reported a study in which dogs were bled and simultaneously infused with a red cell-saline suspension. Their protein fractions were determined both electrophoretically and by chemical fractionation. Results differed somewhat according to the method used, but in both cases the time required for restoration was essentially the same as that observed in the present study. It should be noted that after bleeding and dextran infusion a volume of circulating plasma greater than control volume is maintained for five days.[5] Thus the total quantity of each protein fraction is restored to control level somewhat more quickly than is indicated by concentration data alone. The evidence therefore warrants the conclusion that dextran does not interfere with plasma protein mobilization or regeneration.

## SUMMARY

1. Dogs were severely bled and the blood lost was replaced by an infusion of a 5.7 per cent solution of dextran in saline. Blood samples were taken after bleeding, immediately and one hour after dextran administration, and thereafter at 24-hour intervals for four days. Protein concentrations were determined by micro-Kjeldahl procedures after chemical fractionation.

2. Decreases in albumin concentration are the result of plasma dilution, but evidence is presented that indicates a decrease in fibrinogen concentration greater than can be accounted for by dilution alone.

3. Dextran does not retard protein restoration.

## REFERENCES

1. Ryttinger, L., Swedin, B., and Aberg, B.: The Effect of Dextran on the Erythrocyte Sedimentation Rate in Man, Scandinav. J. Clin. & Lab. Invest. **3**: 221, 1951.
2. Semple, R. E.: Effect of Small Infusions of Various Dextran Solutions on Normal Animals, Am. J. Physiol. **176**: 113, 1954.
3. Thorsén, G.: Nyare unders ökningar över preparatets egenskaper, Nord. med. **40**: 2374, 1948.
4. Wasserman, K., and Mayerson, H. S.: Plasma, Lymph and Urine Studies After Dextran Infusions, Am. J. Physiol. **171**: 218, 1952.
5. Semple, R. E.: The Effects of Single Large Infusions of Various Dextran Solutions on Hypovolaemic Dogs, Can. J. Biochem. & Physiol. **32**: 670, 1954.
6. Hetzel, P. S.: The Clinical Use of Dextran as a Plasma Substitute, M. J. Australia **1**: 657, 1952.
7. Ricketts, C. R.: Interaction of Dextran and Fibrinogen, Nature **169**: 970, 1952.
8. Behrmann, V. G., and Hartmann, F. W.: Studies on Capillary Resistance After Intravenous Infusions, Fed. Proc. **13**: 10, 1954.
9. Smalley, R. E., Binger, M. W., Bollman, J. L., and Power, M. H.: Effect of Intravenously Administered Solution of Acacia on Animals, Arch. Int. Med. **76**: 39, 1945.
10. Gropper, A. L., Raisz, L. G., and Amspacher, W. H.: Plasma Expanders, Surg., Gynec. & Obst. **95**: 521, 1952.
11. Walcott, W. W.: Standardization of Experimental Hemorrhagic Shock, Am. J. Physiol. **143**: 254, 1945.
12. Howe, P. E.: The Determination of Proteins in Blood—A Micro Method, J. Biol. Chem. **49**: 109, 1921.
13. Kingsley, G. R.: A Rapid Method for the Separation of Serum Albumin and Globulin, J. Biol. Chem. **133**: 731, 1940.
14. Cullen, G. E., and Van Slyke, D. D.: Determination of the Fibrin, Globulin and Albumin Nitrogen of Blood Plasma, J. Biol. Chem. **41**: 587, 1920.
15. Zeldis, L. J., and Alling, E. L.: Plasma Protein Metabolism—Electrophoretic Studies, J. Exper. Med. **81**: 515, 1945.

# ANIMAL DISTRIBUTION OF COLLOIDS OF $Au^{198}$, $P^{32}$, AND $Y^{90}$: AN IMPROVED METHOD OF TISSUE ASSAY FOR RADIOACTIVITY

T. N. Lahr, M.S., Robert Olsen, A.B., G. I. Gleason, B.Ch.E., and D. L. Tabern, Ph.D.
North Chicago, Ill.

TO PAUL HAHN and his group[1] belongs the priority for systematically scanning the reactor produced radioisotopes, and selecting $Au^{198}$ as the one most likely to succeed in producing worth-while therapeutic results. Hahn[2] was also the first to suggest that by coating this very stable colloid with inactive silver or silver chloride, its biologic distribution could be significantly altered. A third contribution of this group has been emphasis on the tremendous doses of beta radiation which tissues will tolerate if the material is uniformly distributed in the tissue. In fact, in the dosages of $Au^{198}$ employed today, the 10 per cent of gamma roentgens present is almost sufficient to account for the cancerocidal effects observed, and one has, in addition, the biologic effects of the tenfold greater beta radiation.

One of the reasons for wishing to develop other colloids involving pure $\beta$-emitters is to be able to study independently the clinical value of this beta radiation. A number of such colloids are described in this paper. In evaluating animal results secured with them, one must keep in mind that in animals, gold colloids move from cavities much more rapidly than in human beings, and that, therefore, colloids showing better retention in animals than gold potentially deserve clinical study if other properties are also favorable.[3]

As the result of the pioneering clinical work of Muller[4] and later many others, an entirely new field of application for such radioactive colloids has arisen, i.e., intracavitary administration. Here $Au^{198}$ as gold colloid* is simply infused, as a dilute solution, into the pleural or peritoneal cavity in a rather arbitrarily calculated amount (50 to 150 mc.). The mechanism of action is far from clear, and is, in all probability, multiple in nature. Some of the colloid "sediments" on the wall of the cavity; some is picked up by the lymphatic system and carried to adjacent lymph nodes, or even beyond into the circulation; some, as Goldie and Hahn[5] have shown, is taken up by the phagocytes, and even by free-floating cancer cells, with sedimentation on the area walls. Whether this "coating," which can easily be established by autoradiography, is greater on or near tumor areas has never been definitely proved. At any rate, a number of workers have found that the removal from the cavitary fluid is rapid, and usually complete in forty-eight to seventy-two hours.[3] Best clinical effect, as would be expected, seems to be associated with the prompt deposition on the walls. Since similar "cold" colloids are inactive, it is obvious that the biologic response is due to the radioactive effects. The "average penetration" of the $Au^{198}$ beta,

From the Department of Radioactive Pharmaceuticals, Abbott Laboratories, North Chicago, Ill. and Oak Ridge, Tenn.
Presented to the American Society of Biological Chemists, April 15, 1954, and to the Society of Nuclear Medicine, May 29, 1954.
Received for publication July 30, 1954.
*Aurcoloid (Abbott Laboratories, North Chicago, Ill.).

in tissue, is only 0.9 mm.; beyond this point the energy dose which can be effectively delivered is distinctly limited. Here is where the very penetrating gammas (average half thickness in tissue, 6.6 cm.) come into play. This gamma also permits qualitative scanning from outside the body to study uniformity of distribution. Studies to date show encouragingly widespread placements, and as such scanning devices become more available, this type of "check" will undoubtedly increase in usefulness.

Flocks and associates[6] have shown colloidal $Au^{198}$ to be well retained in the prostate. The rectal irritation sometimes observed at first has been overcome by better techniques and equipment. In a high percentage of cases it has been possible to reduce the prostate tumor to a few small nodules or to cause it to regress even more completely. Negative biopsies in as high as one-third of the cases have been reported, and clinical results after somewhat more than two years continue to look most encouraging. Here, in particular, a $\beta$-emitting colloid would have definite practical advantages, if proved equally effective.

Very interesting also is the use of colloidal gold in an effort to prevent "seeding" of the cavity by tumor cells freed during the operative procedure. Goldie and co-workers[7] have shown that in animals at least, this is certainly theoretically possible, and through clinical experience evidence is gradually accumulating that the same may be true in man. Repetition of these studies using the $\beta$-emitting colloids of this paper is in progress.

Of all the other isotopes currently readily available, $P^{32}$ has the widest background of use. The number of equivalent beta roentgens delivered to tissue per millicurie during its useful life is theoretically some five to ten times that from $Au^{198}$. The prolonged radiation of tumor cells may also have some advantages.

When $P^{32}$ is given in a soluble form, it is rapidly distributed in the body, and, of course, depresses white cell forming areas (the basis for its use in leukemia), so that for fixation in a given area it must be converted into a completely insoluble form. We have studied a number of "chemically insoluble" phosphates, and find that chromic phosphate prepared by a careful precipitation and washing and finally ignition to 400° to 900° C. seems most suitable for clinical use. Such a preparation shows less than 3 to 4 per cent of dialyzable $P^{32}$, and the low excretion values observed (1 to 5 per cent) in our work are further evidence of this point. Since specific activities employed are of the order of 1 to 10 mc. per milligram of $CrPO_4$, any possible toxic action, or cancerocidal side effects, from the chromium, can be ruled out.

For conversion to a clinically useful material, $CrPO_4$ must be mechanically disintegrated. As Gabrieli and colleagues[8] have shown, such suspensions may be far from uniform from batch to batch; particle size also varies within a given lot. The particle size variation increases as the average particle size increases. Dobson and associates[9] have resorted to elaborate sedimentation procedures to select those rapidly sedimenting particles necessary for these liver-clearance studies.

When used for attempting to irradiate a body cavity or tissue uniformly, exactly the opposite type of colloid is needed. It has been calculated that 1 c.c. of gold colloid (particle size 0.003 to 0.007 $\mu$) contains several billion particles, each of which, if uniformly dispersed, acts as a point source. The larger chromic phosphate (0.2 to 2.0 $\mu$) contains far fewer such point sources. We have, therefore, in this work studied the incorporation of various colloids to achieve maximal disintegration and still maintain retention, as has been done for the gold colloid. The effect of surface active agents has also been studied.

A potentially even more interesting $\beta$-emitting isotope is $Y^{90}$ whose average tissue penetration is 3.5 mm. $Y^{90}$ is a daughter of $Sr^{90}$, and if we adsorb $Sr^{90}$ on a column, we can take off same 75 per cent of the equivalent $Y^{90}$ activity each week with no serious reduction in the amount of $Sr^{90}$ remaining. Several factors make this look attractive, but the highly hazardous nature of the parent strontium and the possibility of traces remaining in the separated yttrium poses a very real safety problem. For instance, decomposition of the solvent and of the column material itself by radiation results in mechanical failures. Each batch, after repurification on a second column, must be tested, and $Sr^{90}$ shown to be completely absent.

Yttrium$^{90}$ belongs to a unique class of elements capable of forming "radio colloids." For instance, as Schweitzer[10] has shown, if a diluted solution of carrier-free $YCl_3$ is brought to a pH of 7 to 8, simple filtration results in a complete retention of activity on the filter. In biologic systems, the situation is far more complex. For instance, Kawin[11] has recently shown that $Y^{90}$ also forms stable complexes with low molecular weight proteins and protein fragments, which can be chromatographed as such by the use of several widely differing solvents. We have found that stable combinations are formed betweeen $Y^{90}$ and simple nucleic acids, which are always present to a greater or lesser extent in fluid accumulations. Simon has recently observed that even gold colloid rapidly associates with proteins, migrating durating electrophoresis with the $\alpha$ and $\beta$ globulins, but not with the albumins.[12] Two relatively insoluble yttrium colloids have also been investigated in this work, i.e., the phosphate and the fluoride.

Biologic application of $Y^{90}$ has been retarded by earlier reports that, in animal experiments, a considerable portion goes to the bone and to the reticuloendothelial system. We have found that, as Lewin and associates[13] have just reported, by a proper selection of colloid form, of pH, and of additional carrier, these difficulties can be minimized.

Simultaneously, studies have been made an these same lots of $Y^{90}$ solution by Dr. Kyker at ORINS, and by Dr. G. V. LeRoy at the University of Chicago; these studies will be reported elsewhere.[14]

## MATERIALS AND METHODS

In this work over four hundred animals have been used. The average number per value reported was eight. Many represent the use of at least two and sometimes as high as eight different preparations of colloid, thus minimizing errors due to variation in preparation, or properties.

*Compounds Studied in This Investigation.—*

*$Au^{198}$ gold colloid*:* This colloid was prepared from gold foil irradiated in the ORNL reactor. It was a cherry-red liquid, with a particle size of 0.003 to 0.007 $\mu$ and specific activity of 3 to 5 mc. per milligram at the time of preparation.[15] The rat dose of this and all other gold colloids studied was 500 $\mu$c in a volume of 0.3 to 1.0 ml.

*$Au^{198}$ high specific activity gold colloid:* This colloid had essentially the same physical characteristics as the regular gold colloid but was prepared from gold foil irradiated in a higher neutron flux and had a specific activity of from 10 to 15 mc. per milligram.

*$Au^{198}$ silver on gold colloid:* This colloid was prepared by the precipitation of metallic silver on the high specific activity gold colloid, essentially according to the method of Hahn.[2]

*$P^{32}$ chromic phosphate ($CrP^{32}O_4$)—Type G:* This was made by a modification of the method of Ness and collaborators.[16] The precipitated compound was ignited at 400° to 600° C. to give anhydrous $CrP^{32}O_4$, which was ground to a particle size of 0.05 to 1.0 $\mu$.* The material was suspended in 30 per cent glucose solution containing 0.5 per cent phenol as preservative. The specific activity was 1 to 10 mc. per milligram and the rat dose was 300 $\mu$c in 0.2 to 1.0 ml. of solution. These conditions of specific activity and and dosage were the same in all of the modified preparations to be outlined in this paper.

*$P^{32}$ chromic phosphate ($CrP^{32}O_4$) in pectin:* This material was precipitated and ignited as above but was ground to a particle size of 2 to 4 $\mu$ and was suspended in 2 per cent pectin.

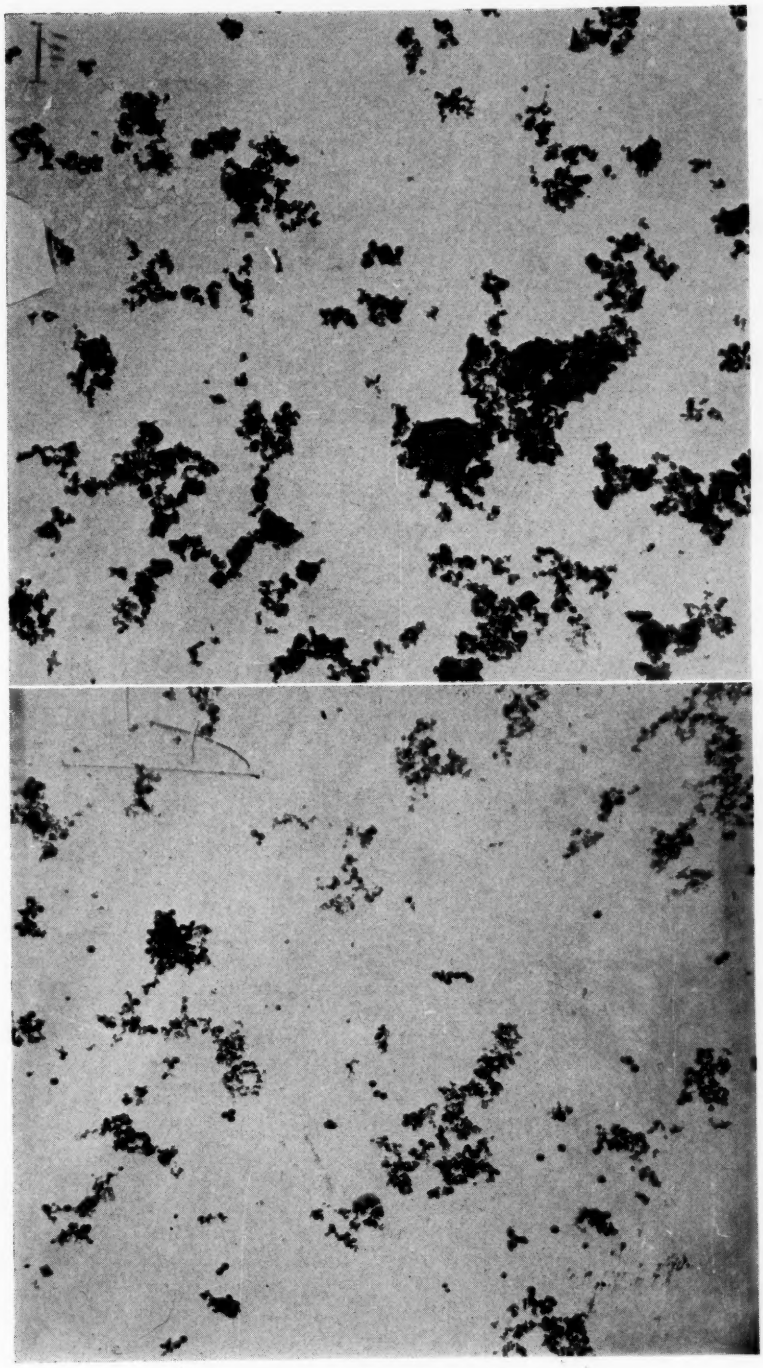
*$P^{32}$ chromic phosphate in gelatin:* This material was prepared and ignited as above but was ground to a particle size of less than 0.4 $\mu$ and suspended in 4 per cent gelatin.

*$P^{32}$ chromic phosphate (Libby):* This material was kindly supplied by Dr. Raymond L. Libby of the Veterans Hospital, Los Angeles, California.

*$P^{32}$ titanium phosphate-germanium dioxide ($Ti_3[PO_4]_4GeO_2$):* This material was made available by Commander H. C. Dudley, Naval Hospital, St. Albans, L. I., New York.

*Chromic phosphate has a remarkable tendency to condense into nontransparent aggregates that appear under the microscope to have a particle size of 0.4 to 3.0 $\mu$. Examination of a fully dispersed Type G in the electron microscope shows it to be composed of ultimate units much smaller than this, namely, between 0.05 and 1.0 $\mu$. Some data indicate that when phagocytosed, partial agglomeration takes place in the cell just as is the case with gold.

*A.*



*B.*

Fig. 1.—*A*, $CrP^{32}O_4$ in pectin. *B*, Type "G" $CrP^{32}O_4$.

*$Y^{90}$ yttrium chloride ($Y^{90}Cl_3$):* We are preparing this compound by a chromatographic separation from $Sr^{90}$ and it has been found to contain less than one part per million of $Sr^{90}$ by a chromatographic method devised by one of us (G. I. Gleason). The compound was prepared in aqueous solution at pH 4 and the specific activity was 0.5 to 1.5 mc. per milligram of Y at the time of injection. The rat dose was 200 $\mu$c for all $Y^{90}$ compounds.

*$Y^{90}$ yttrium phosphate ($Y_2^{90}[HPO_4]_3$):* This compound was prepared as a gelatinous precipitate by the addition of $Na_2HPO_4$ solution to the previously described $Y^{90}Cl_3$ solution followed by thorough washing. It was not ignited. The specific activity was, of course, the same as the $Y^{90}Cl_3$. The material was injected in either aqueous or 30 per cent glucose solution. No difference between these media could be detected and so no differentiation is made in the tables of results.

*$Y^{90}$ yttrium fluoride ($Y^{90}F_3$):* The material was prepared by the addition of carrier Y to the $Y^{90}Cl_3$ solution and precipitation with NaF. The specific activity was 0.06 mc. per milligram and the gelatinous precipitate was suspended in 30 per cent glucose solution.

The rats used were white Wistar strain, of both sexes, and an average weight of 150 grams. The intramuscular injections were made into the gluteal muscle, and the intravenous into the tail vein. The intraperitoneal injections were made directly into the peritoneal cavity, with special care being taken to avoid the intestine. The intrapleural injections were made directly into the thoracic cavity, following the injection of a somewhat larger volume of air to avoid intravenous deposition.

At the time of injection an equal volume of the compound was measured into a 100 ml. volumetric flask with the same syringe used for the animal injection. This was then diluted to the mark with a suitable solvent, usually water or dilute HCl, though in the case of the $Au^{198}$ colloids, the aliquot was dissolved in a few milliliters of aqua regia and then diluted to 100 ml. with water. This standard made the calculation of the data very simple and eliminated the need for a decay correction.

The animals were kept in metabolism cages and the combined urine and feces were homogenized and counted to determine the amount of isotope excreted. The values for excretion are not reported in the data since they were only 1 to 2 per cent per week in the great majority of the cases.

The rats were sacrificed after five to seven days. We were unable to find any differences between animals that lived five days and those that lived seven. Other data, not here reported, lead us to believe that the majority of translocation takes place in 48 hours or less.

At autopsy the liver, spleen, both kidneys, both lungs, and both femurs of each animal (except those injected intrapleurally where the entire thorax was removed instead of the lungs) were dissected out and the entire organ or organs homogenized in a Potter-Elvejhm homogenizer or a Waring Blendor. An equal volume of formamide was added to the aqueous tissue homogenate to give a clear to somewhat cloudy solution.[17] The thoraces and femurs were not dispersed well by this technique and so were dissolved by treatment with concentrated nitric acid. Bone marrow samples were taken by mechanical removal from some of the femurs.

Twenty milliliters of the tissue homogenate, or nitric acid solution, or a suitable aliquot diluted to 20 ml., was then placed in a 30 ml. Pyrex beaker and liquid counted, at a reproducible geometry, using a Nuclear-Chicago D34 Geiger detector and a Nuclear-Chicago 1615B ratemeter. We have found this method to be simple, quick, reproducible, and unaffected by the amount of tissue in the counting sample. Since the amount of solution used constituted infinite thickness for the beta particles of $Au^{198}$, $P^{32}$, and $Y^{90}$, small errors in measurement of sample volume did not change the counting rate observed appreciably. By counting a suitable aliquot of the standard prepared at the time of injection, we determined the total CPM in the animal and could calculate our data directly as a percentage of the injected dose. Some of the $Au^{198}$ samples were counted in a Welch-Allyn "Texas Well" well-type Geiger counter using the 1615B ratemeter as the recording device. On those animals where we attempted to account for the entire dose, recoveries usually were 90 to 95 per cent.

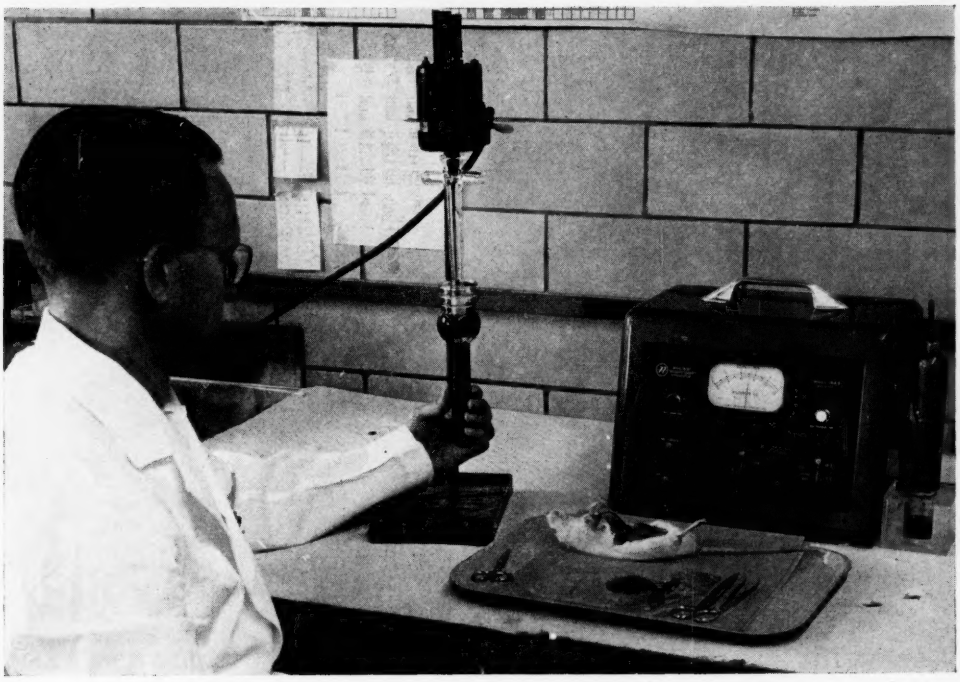


Fig. 2.—Equipment used for assay of radiocolloids deposited in tissues.

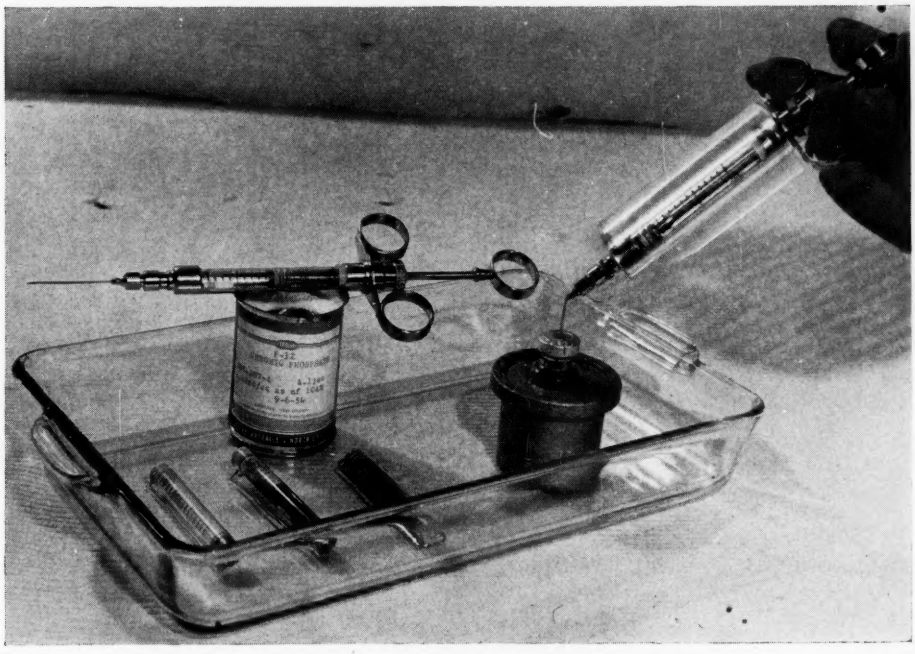


Fig. 3.—The "Jaffe" syringe for handling $\beta$-emitting isotopes.

TABLE I. RATS—INTRAPERITONEAL INJECTION

| ISOTOPE | $Au^{198}$ | $Au^{198}$ | $Au^{198}$ | $P^{32}$ | $P^{32}$ | $P^{32}$ | $P^{32}$ | $P^{32}$ | $Y^{90}$ | $Y^{90}$ | $Y^{90}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| COMPOUND | GOLD COLLOID | HIGH SPECIFIC ACTIVITY GOLD COLLOID | SILVER ON GOLD COLLOID | $CrPO_4$ TYPE G | $CrPO_4$ PECTIN | $CrPO_4$ GELATIN | $CrPO_4$ LIBBY | $Ti(PO_4)_4$ $GeO_2$ | $YCl_3$ | $Y_2(HPO_4)_3$ | $YF_3$ |
| NO. ANIMALS | 9 | 8 | 5 | 19 | 20 | 3 | 4 | 3 | 28 | 16 | 2 |
| Liver | 26.8 | 19.5 | 38.4 | 8.8 | 22.6 | 61.5 | 11.7 | 11.4 | 21.1 | 11.1 | 11.4 |
| Spleen | 1.3 | 4.9 | 4.0 | 1.8 | 3.9 | 1.5 | 3.8 | 2.2 | 4.2 | 4.0 | 8.3 |
| Kidney | 0.02 | 0.8 | 1.7 | 0.1 | 0.2 | 0.08 | 0.3 | 0.01 | 0.6 | 0.3 | 0.4 |
| Lung | 0.01 | 1.1 | 4.6 | 0.2 | 0.06 | 0.06 | 0.2 | 0.2 | 0.6 | 0.6 | 1.2 |
| Bone marrow | 0.01 | – | – | 0.005 | 0.01 | – | – | – | – | – | – |
| Femurs | – | 0.18 | 0.20 | 0.35 | – | – | 0.38 | 0.83 | 0.56 | 0.42 | 0.80 |

TABLE II. EFFECT OF VARIOUS COMPOUNDS ON UPTAKE OF COLLOIDS IN RATS ON INTRAPERITONEAL INJECTION

| ISOTOPE | $P^{32}$ | $P^{32}$ | $P^{32}$ | $P^{32}$ | $Y^{90}$ | $Y^{90}$ | $Y^{90}$ | $Y^{90}$ | $Y^{90}$ |
|---|---|---|---|---|---|---|---|---|---|
| COMPOUND | $CrPO_4$ TYPE G | $CrPO_4$ + ASCITIC FLUID | $CrPO_4$ + DUPANOL* PC | $CrPO_4$ + ASCITIC FLUID + DUPANOL PC | $YCl_3$ | $YCl_3$ + ASCITIC FLUID | $YCl_3$ + RAT PLASMA | $Y_2(HPO_4)_3$ | $Y_2(HPO_4)_3$ ASCITIC FLUID |
| NO. ANIMALS | 19 | 4 | 3 | 4 | 28 | 7 | 3 | 16 | 8 |
| Liver | 8.8 | 20.8 | 18.1 | 27.4 | 21.1 | 21.3 | 28.1 | 11.1 | 14.2 |
| Spleen | 1.8 | 2.7 | 2.8 | 2.9 | 4.2 | 12.4 | 7.8 | 4.0 | 7.7 |
| Kidney | 0.1 | 0.3 | 0.2 | 0.2 | 0.6 | 0.5 | 0.6 | 0.3 | 0.4 |
| Lung | 0.2 | 0.5 | 3.1 | 1.3 | 0.6 | 0.4 | 0.9 | 0.6 | 1.0 |
| Femurs | 0.35 | 0.44 | 0.34 | 0.40 | 0.56 | 1.41 | 0.90 | 0.42 | 1.06 |

*One-tenth per cent sodium lauryl sulfate.

### HANDLING PROCEDURES FOR $\beta$-EMITTING COLLOIDS

While the amounts of activity used in these small animals did not specifically require special shielding of syringes, the clinical trial of these $\beta$-emitting colloids as suggested by this work, and currently underway, made a study of handling methods desirable. A cartridge type of Lucite-shielded syringe has, therefore, been developed in collaboration with Dr. Henry Jaffe.*

In this, the sterile solution or suspension is loaded into a special 3 ml. cartridge, along with vasoconstrictors, hyaluronidase, etc. Since each cartridge may be made to contain up to 6 mc., one, or at the most two, will suffice for the average case.

At the conclusion of the injection, the cartridge is simply removed and discarded, reducing contamination and handling of "hot" equipment. The 5/8-inch Lucite shield is sufficient to keep radiation well within tolerance levels without interfering with accurate reading of the calibrations.

### RESULTS

*Intraperitoneal Injection.*—Table I shows the distribution of the various compounds when injected intraperitoneally. The values given are simple averages and are expressed as a percentage of the injected dose.

Type G $CrPO_4$ transport, as measured by pickup in the liver, spleen, and femurs, was the lowest observed among the many preparations tested.

To estimate skeletal pickup the two femurs still containing the bone marrow were assayed as above. Since the femurs constitute about 10 per cent of the rat skeleton, one should multiply the values in Table I by this factor to secure an approximate skeletal content. Certainly results with the various preparations may be compared on this basis. Some 2 per cent of the gold dose, 3.5 to 4.0 per cent of Type G $CrPO_4$ and 5.0 to 8.0 per cent of the Y have been thus calculated to be in the skeleton following intraperitoneal administration.

It seems that the particle size is definitely less important than the organic coating around the particle in the case of $CrP^{32}O_4$. For example, $CrP^{32}O_4$ in gelatin has most of its particles in the same size range as $CrP^{32}O_4$ Type G. However, the liver pickup is 61.5 per cent as opposed to 8.8 per cent for the material in 30 per cent glucose. Also, $CrP^{32}O_4$ in pectin, which has a much larger particle size, might, therefore, be expected to be retained better, but actually is found in the liver to an extent of 22.6 per cent, 2.5 times the $CrP^{32}O_4$ Type G.

$CrP^{32}O_4$ as prepared by Dr. Libby, $Ti_3(P^{32}O_4)_4GeO_2$, $Y_2^{90}(HPO_4)_3$, and $Y^{90}F_3$ have distributions quite similar to $CrP^{32}O_4$ Type G (but seem to be deposited more in the spleen and femurs).

$Y^{90}Cl_3$ and the three $Au^{198}$ colloids are quite similar to each other in liver and spleen pickup but almost three times as much of the $Y^{90}$ compound can be found in the femurs.

The striking effect of these "organic coatings" on the distribution of these compounds can be seen in Table II, where the data are tabulated from a series of experiments in which human ascitic fluid, 0.1 per cent sodium lauryl sulfate, and rat plasma were added before injection to some of the compounds under study.

*Pilot models are being made on order.

TABLE III. MICE—INTRAPERITONEAL INJECTION

| ISOTOPE | $Au^{198}$ | $Au^{198}$ | $Au^{198}$ | $P^{32}$ | $P^{32}$ | $P^{32}$ | $Y^{90}$ |
|---|---|---|---|---|---|---|---|
| COMPOUND | AURCOLOID | HIGH SPECIFIC ACTIVITY AUROCLOID | SILVER ON GOLD COLLOID | $CrPO_4$ TYPE G | $CrPO_4$ PECTIN | $CrPO_4$ GELATIN | $YCl_3$ |
| NO. ANIMALS | 5 | 6 | 8 | 37 | 5 | 5 | 9 |
| Liver | 31.1 | 22.8 | 24.6 | 8.1 | 36.7 | 51.0 | 17.7 |
| Spleen | 2.3 | 4.4 | 4.4 | 2.7 | 3.2 | 3.9 | 2.3 |
| Carcass | 65.7 | 66.5 | 62.9 | 64.0 | 47.4 | 32.0 | 60.9 |

TABLE IV. RATS—INTRAVENOUS INJECTION

| ISOTOPE | $Au^{198}$ | $Au^{198}$ | $Au^{198}$ | $P^{32}$ | $P^{32}$ | $P^{32}$ | $Y^{90}$ | $Y^{90}$ | $Y^{90}$ |
|---|---|---|---|---|---|---|---|---|---|
| COMPOUND | GOLD COLLOID | HIGH SPECIFIC ACTIVITY GOLD COLLOID | SILVER ON GOLD | $CrPO_4$ TYPE G | $CrPO_4$ PECTIN | $CrPO_4$ GELATIN | $YCl_3$ | $Y_2(HPO_4)_3$ | $YF_3$ |
| NO. ANIMALS | 4 | 4 | 4 | 11 | 22 | 6 | 11 | 4 | 4 |
| Liver | 88.7 | 95.8 | 93.9 | 77.0 | 63.2 | 63.7 | 40.5 | 19.8 | 29.2 |
| Spleen | 1.8 | 1.0 | 1.9 | 3.0 | 11.6 | 14.4 | 5.9 | 35.5 | 44.8 |
| Kidney | 0.1 | 0.1 | 0.8 | 0.1 | 0.1 | 0.3 | – | 0.7 | 0.7 |
| Lung | 0.2 | 0.2 | 0.1 | 1.5 | 0.5 | 0.7 | 0.7 | 7.2 | 5.7 |
| Bone marrow | – | – | – | 0.004 | 0.009 | 0.01 | – | – | – |
| Femurs | 0.24 | 0.21 | 0.34 | – | – | – | 1.2 | 1.8 | 2.1 |

The addition of either 0.1 per cent sodium lauryl sulfate or human ascitic fluid to $CrP^{32}O_4$ Type G caused a greatly increased liver pickup, while spleen and bone deposition was increased only slightly. The two compounds have an additive effect as can be seen in the case where both were added to $CrP^{32}O_4$ Type G before injection to give a liver pickup three times that of $CrP^{32}O_4$ Type G alone. In the case of the ascitic fluid, a "coating" similar to that of pectin or gelatin may account for the increased pickup, while the sodium lauryl sulfate (a dispersing and surface-active agent) may disperse the loosely agglomerated chromic phosphate particles in the body fluids causing increased translocation.

In the case of $Y^{90}Cl_3$, the addition of human ascitic fluid before injection did not affect the liver deposition significantly but almost tripled the amount going to the spleen and femurs. Even the rat's own plasma had this effect, although to a lesser degree. $Y_2{}^{90}(HPO_4)_3$ also exhibited this behavior when injected along with human ascitic fluid. The mechanism for this altered distribution is not clear, but we have found by dialysis experiments that $Y^{90}$ is bound, in vitro, quite firmly to human ascitic fluid, rat plasma, and ribonucleic acid.

To study the possibility of species differences, mice were injected intraperitoneally (Table III) with each of these principal colloids, and liver and spleen pickup were studied. The values attained paralleled quite closely those obtained in the rat. As a rough check on total recoveries, the remainder of the carcass was homogenized and assayed for activity. With the exception of one series, recoveries of 80 to 99 per cent were observed.

*Intravenous Injection.*—As can be seen in Table IV, the distribution of the $Au^{198}$ and $P^{32}$ compounds upon intravenous administration followed the expected pattern, that is, 80 to 100 per cent of the material was found in the liver, spleen, and lungs, with most of the isotope localizing in the liver.

The distribution of $Y_2{}^{90}(HPO_4)_3$ and $Y^{90}F_3$, however, was extremely interesting and quite unexpected. The spleen in each case contained much more $Y^{90}$ than the liver, on an isotope per gram basis 8 to 15 times as much. The skeletal deposition was calculated to be about 20 per cent and a very significant amount was found in the lungs. Apparently, the compounds were quite selectively localized in the blood-forming organs, the spleen especially receiving large amounts of the beta radiation.

*Intrapleural Injection.*—The thorax, including the rib cage and the diaphragm, is easily dissected out intact and can be dissolved and counted, giving a quantitative measure of the retention of the isotope within the cavity and, conversely, the amount translocated from the space. Theoretically, the more isotope retained within the cavity, the greater should be the efficiency in alleviating pleural fluid accumulation and also the less should be the undesirable effects associated with radiation of other body organs. In Table V are tabulated the data obtained from the autopsy of rats injected intrapleurally with some of the compounds undergoing study.

All of the $P^{32}$ and $Y^{90}$ compounds studied were about 80 to 90 per cent retained within the pleural space. All three of the $Au^{198}$ colloids are less well

TABLE V. RATS—INTRAPLEURAL INJECTION

| ISOTOPE | $Au^{198}$ | $Au^{198}$ | $Au^{198}$ | $P^{32}$ | $P^{32}$ | $P^{32}$ | $P^{32}$ | $Y^{90}$ | $Y^{90}$ | $Y^{90}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| COMPOUND | GOLD COLLOID | HIGH SPECIFIC ACTIVITY GOLD COLLOID | SILVER ON GOLD COLLOID | $CrPO_4$ TYPE G | $CrPO_4$ PECTIN | $CrPO_4$ LIBBY | $Ti_3(PO_4)_4$ $GeO_2$ | $YCl_3$ | $Y_2(HPO_4)_3$ | $YF_3$ |
| NO. ANIMALS | 7 | 4 | 4 | 12 | 5 | 3 | 4 | 11 | 5 | 4 |
| Thorax | 47.3 | 66.3 | 62.0 | 89.3 | 78.6 | 89.8 | 89.5 | 81.8 | 82.3 | 87.7 |
| Liver | 36.7 | 30.2 | 32.2 | 5.8 | 6.0 | 7.1 | 2.5 | 2.8 | 5.1 | 3.2 |
| Spleen | 0.6 | 0.6 | 1.4 | 0.3 | 0.5 | 0.4 | 0.3 | 0.8 | 0.4 | 3.1 |
| Kidney | 0.3 | 0.2 | 0.7 | 0.04 | – | 0.05 | – | 0.01 | 0.08 | 0.09 |
| Bone marrow | – | – | – | – | 0.001 | – | – | – | – | – |
| Femurs | 0.26 | 0.1 | 0.2 | 0.25 | – | 0.32 | 0.71 | 0.06 | 0.16 | 0.17 |

retained, 30 to 40 per cent being found outside of the thorax. If the factor of 10 for skeleton deposition is applied to the femur values, and the excretion, which is not reported, is taken into account, our average recovery is about 95 per cent of the injected isotope.

TABLE VI. RABBITS—INTRAPLEURAL INJECTION

| ISOTOPE | $P^{32}$ | $Au^{198}$ | $Y^{90}$ |
|---|---|---|---|
| COMPOUND | $CrPO_4$ TYPE G | AURCOLOID | $YCl_3$ |
| NO. ANIMALS | 3 | 3 | 3 |
| Thorax | 89.2 | 59.9 | 84.6 |
| Liver | 2.4 | 33.3 | 0.5 |
| Spleen | 0.1 | 0.4 | 0 |
| Kidney | 0 | 0.3 | – |
| Bone marrow | 0 | 0.003 | – |
| Femur | – | – | 0.3 |

Several rabbits were also injected intrapleurally (Table VI) with $Au^{198}$ gold colloid, $CrP^{32}O_4$ Type G and $Y^{90}Cl_3$. Essentially the same distribution was observed as in the rat, 85 to 90 per cent remaining in the thorax in the case of $CrP^{32}O_4$ Type G and $Y^{90}Cl_3$ and only about 60 per cent in the case of $Au^{198}$ gold colloid.

*Intramuscular Injection.*—Table VII shows the data obtained when the various compounds were injected into the gluteal muscle of rats. Less than 2 per cent total translocation was observed in all cases.

TABLE VII. RATS—INTRAMUSCULAR INJECTION

| ISOTOPE | $Au^{198}$ | $Au^{198}$ | $Au^{198}$ | $P^{32}$ | $P^{32}$ | $Y^{90}$ | $Y^{90}$ |
|---|---|---|---|---|---|---|---|
| COMPOUND | GOLD COLLOID | HIGH SPECIFIC ACTIVITY GOLD COLLOID | SILVER ON GOLD COLLOID | $CrPO_4$ TYPE G | $CrPO_4$ PECTIN | $YCl_3$ | $Y_2(HPO_4)_3$ |
| NO. ANIMALS | 4 | 4 | 4 | 11 | 16 | 3 | 4 |
| Liver | 0.8 | 0.1 | 0.5 | 0.3 | 0.9 | 0.7 | 0 |
| Spleen | 0.1 | 0.01 | 0.06 | 0.03 | 0.06 | 0.03 | 0 |
| Kidney | 0.2 | 0.2 | 1.0 | 0.03 | 0.02 | 0.02 | 0 |
| Lung | 0.6 | 0.02 | 0.06 | 0.03 | – | 0.04 | 0 |
| Bone marrow | 0.08 | – | – | 0.003 | 0.001 | – | – |
| Femur | – | – | – | – | – | 0.03 | 0.03 |

## DISCUSSION

As indicated earlier, in interpreting this data, one must clearly recognize the inherent differences between the biologic processes going on through the walls of the pleural and peritoneal cavities of these small, normal animals, and those found in the human being in the presence of extensive metastatic tumor growth. An analogous situation has recently been reported by Bangham and associates.[18] Using a colloidal radioactive glass, they were able to show that the normal peritoneal membrane of animals is relatively permeable to "smooth" particles, but that the use of "sharp" particles, or a chemical irritant, quickly upsets the normal state and prevents transfer of the colloid from the cavity. The fact that in the clinical case there is fluid accumulation

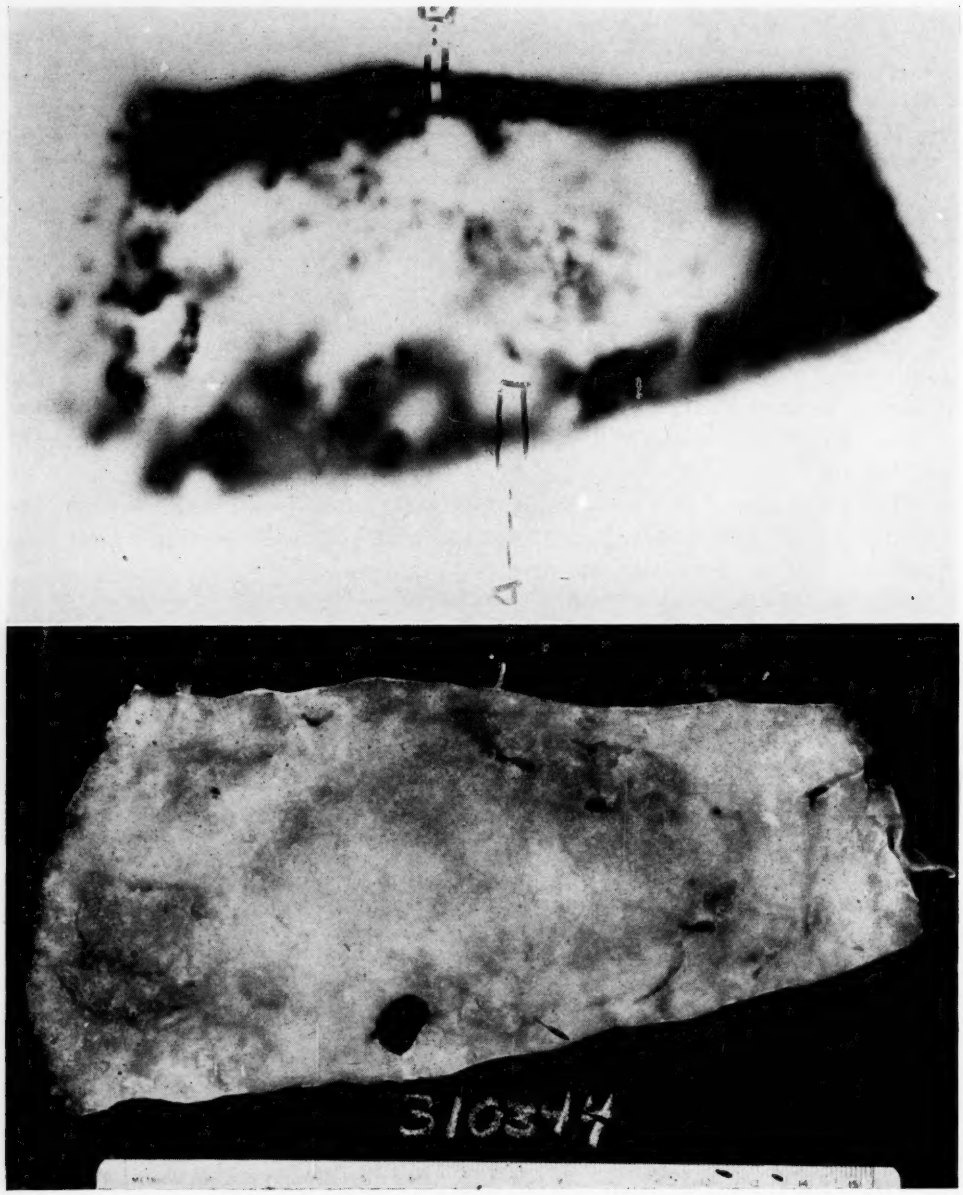



Fig. 4.—Radiograph (top) and photograph of cancerous parietal pleura from patient treated with $CrP^{32}O_4$. Note uneven distribution.

is, in itself, good evidence that the permeability of the walls and the functioning of the lymphatic system has been markedly altered by the presence of the tumor.

Andrews and co-workers,[3] among others, have emphasized that the fall in the amount of gold activity in the cavitary fluid to a half value in two to three days represents not a transfer from the cavity, but rather, for the most part, a fixation on the superficial portions of the available serous surfaces. This has been well authenticated by autoradiograms and by tissue assays. External counting assays over the liver indicate that there is not a prominent translocalization to this organ in human cases as might be inferred from this animal work.

In the case of short-lived isotopes, like $Au^{198}$, the amount accumulating in the liver and spleen may not be of primary significance. In the treatment of leukemias with intravenous gold, Hahn has cited many cases which must have 20 to 40 mc. there, and yet have only temporary depression of the blood picture.

Studies such as ours do not answer one important question: what is the degree and evenness of colloid deposition on those surfaces or in those areas of tumor patients who require irradiation? What evidence is available, most as yet unpublished, indicates the distribution of $CrPO_4$ is not as uniform, under these conditions, as is that of $Au^{198}$ colloid. Fig. 4 shows the autoradiogram of a section of parietal pleura taken at autopsy of a patient who had received colloidal chromic phosphate fourteen days before death*; obviously in this case there was less deposition on the central tumorous area than on the surrounding areas.

## CONCLUSIONS

A quantitative comparison is presented of three types of chromic phosphate colloids, and of several colloids prepared from $Au^{198}$ and $Y^{90}$. The data indicate excellent cavitary retention in the case of Type G $CrPO_4$, and excellent retention of $Y^{90}$, as the phosphate. In view of the established clinical safety of $Au^{198}$ colloid (Aurcoloid), several of these others are undergoing clinical evaluation by groups with experience in this field. Data regarding cavitary distribution and clinical effectiveness can be secured only on suitable clinical material. Also presented is a much simplified method for tissue assays, involving homogenization of whole organs, solution of aliquots at room temperature in Formamide, and either beta counting at infinite thickness, or, where possible, gamma counting in a suitable well-type scintillation unit.

## ADDENDUM

Since the submission of this manuscript, one excellent article involving some of the preparations used in our work has appeared—McCormick, James B., Milles, George, Jaffe, Bertha, and Seed, Linton: Tissue Distribution of Injected Radioactive Colloidal Chromic Phosphate ($CrP^{32}O_4$), Arch. Path. 58: No. 3, 187, 1954.

*Supplied by Dr. R. G. Andrews, Medical Department, ORINS, Oak Ridge, Tenn.

REFERENCES

1. Hahn, P. F., and Sheppard, C. W.: Selective Radiation Obtained by the Intravenous Administration of Colloidal Radioactive Isotopes in Diseases of the Lymphoid System, South. M. J. **39:** 558-62, 1946.
2. Hahn, P. F., and Carothers, E. L.: Radioactive Metallic Gold Colloids Coated With Silver and Their Distribution in the Lung and Its Lymphatics Following Intrapulmonary Administration: Therapeutic Implications in Primary Lung and Bronchiogenic Tumors, Brit. J. Cancer **5:** 400-4, 1951.
3. (a) Andrews, G. A., Root, S. W., and Kniseley, R. M.: Metabolism and Distribution of Colloidal $Au^{198}$ Injected Into Serous Cavities for Treatment of Effusions Associated With Malignant Neoplasma, Cancer **6:** 294, 1953.
   (b) Kniseley, R. M., and Andrews, G. A.: Pathologic Changes Following Intercavity Therapy With Colloidal Gold, Cancer **6:** 303, 1953.
   (c) Stembridge, V. A., Kniseley, R. M., and Gibbs, W.: Autoradiographic Distribution of Intravenous Colloidal Gold ($Au^{198}$) in the Rat, Lab. Invest. **2:** 349, 1953.
4. Muller, J. H.: Weitere entwicklung der therapie von peritoneal-carcinosen bei ovarialcarcinom mit kunstilcher radioaktivitat ($Au^{198}$), Gynaecologica **129:** 289-94, 1950.
5. Goldie, H., and Hahn, P. F.: Distribution and Effect of Colloidal Radioactive Gold in Peritoneal Fluid Containing Free Sarcoma 37 Cells, Proc. Soc. Exper. Biol. & Med. **74:** 638-42, 1950.
6. Flocks, R. H., Kerr, H. D., Elkins, H. B., and Culp, D.: Treatment of Carcinoma of the Prostate by Interstitial Radiation With Radioactive Gold ($Au^{198}$): A Preliminary Report, J. Urol. **68:** 510-22, 1952; Ibid. **71:** 628, 1954.
7. Goldie, H., West, H. D., Jeffries, B. R., and Butler, C. H.: Intrapleurally Injected Colloidal $Au^{198}$ and Growth Implantation of Free Tumor Cells in Pleural Exudate, Proc. Soc. Exper. Biol. & Med. **80:** 327-31, 1952.
8. Gabrieli, E. R.: Velocity at Which Radioactive Colloids Disappear From Blood. Studies on Function of Reticulo-Endothelial System, Acta physiol. scandinav. **23:** 283, 1951.
9. (a) Dobson, E. L., Gofman, J. W., Jones, H. B., Kelly, L. S., and Walker, L. A.: The Controlled Selective Localization of Radioisotopes of Yttrium, Zirconium and Columbium in the Bone Marrow, Liver and Spleen, J. LAB. & CLIN. MED. **34:** 297-312, 1949.
   (b) Jones, H. B., Wrobel, C. J., and Lyons, W. R.: A Method of Distributing B-Radiation to the Reticulo-endothelial System and Adjacent Tissues, J. Clin. Invest. **23:** 783, 1944.
   (c) Gofman, J. W.: The Chemical Principles and Methods Involved in Preparation of Colloids of Yttrium, Zirconium, Columbium and Lanthanum, J. LAB. & CLIN. MED. **34:** 297-312, 1949.
10. Schweitzer, G. K., Stein, B. R., and Jackson, W. M.: Low Concentration Chemistry. III. The Radiocolloidal Properties of $Yttrium^{90}$, J. Am. Chem. Soc. **75:** 793, 1953.
11. Kawin, B.: Metabolism of Radioyttrium, Arch. Biochem. **45:** 230, 1953.
12. Simon, N.: Radioactive Gold in Filter Paper Electrophoresis Patterns of Plasma, Science **119:** 95, 1953.
13. Lewin, B., et al.: Distribution of $Y^{90}$ in Ascites Tumor Mice Following Intraperitoneal Administration of Yttrium Chloride, Science **119:** 880, 1954.
14. Kyker, G. C.: Biochemical Consideration of Distribution, Am. J. M. Sc. **227:** 572-88, 1954.
15. Tabern, D. L.: Administration and Uses of Radioactive Colloidal $Gold^{198}$, 1953.
16. Ness, A. T., Smith, R. E., and Evans, R. L.: The Preparation and Some Properties of Chromic Phosphate, J. Am. Chem. Soc. **74:** 4,685-88, 1952.
17. Tabern, D. L., and Lahr, T. N.: A Simplified Method for Determining Radioisotopes in Tissue, Science **119:** 739-40, 1954.
18. Bangham, A. D., Magee, P. M., and Osborn, S. B.: The Effect of Inflammation and Other Factors on the Movement of Radioactive Glass Particles From the Peritoneal Cavity, Brit. J. Exper. Path. **34:** 1, 1953.

# THE IMMUNOLOGIC RESPONSE OF MICE FROM STOCKS RESISTANT AND SUSCEPTIBLE TO ACUTE DISSEMINATED ENCEPHALOMYELITIS

PETER K. OLITSKY, M.D., AND JOHANNA M. LEE, PH.D.
NEW YORK, N. Y.

REFERENCE has already been made to the resistant (RR) and susceptible (SS) stocks of mice of certain genotypic constitution, to the development of the acute disseminated encephalomyelitis (ADE) which follows inoculation of normal brain tissue or its proteolipid component mixed with a Freund-type adjuvant.[1-4] It was suggested that a heritable factor, as dominant, governs the resistance[4] and that the basis of the ADE reaction is the development of hypersensitivity to the cerebral tissue.[3] The problem now is whether the RR and the SS stocks show any difference in their immunologic reactivity as found by means of the techniques described in this paper; an additional experiment will be included in the report of these results, on the presence of encephalitogenic proteolipid in the brain of RR mice, as has already been shown for the SS breed.

## MATERIALS AND METHODS

The procedures for tests of the reactivity of RR and SS mice comprised: (1) serum agglutination in both types of animals that were immunized with mixed *S. typhosa* and *S. paratyphi A + B;* (2) serum precipitation with horse serum, also with crystalline bovine plasma albumin (BPA) as immunizing and test antigens; (3) sensitization with BPA for the respective anaphylaxis reactions; (4) passive transfer of selected rabbit antisera to find out if passive anaphylaxis would thereby be induced in these breeds of mice.

For the agglutination test, 20 mice each of RR and SS breeds were immunized with 6 injections of the standard TAB vaccine of the New York City Health Department. Each mouse received subcutaneously 0.1, 0.2, and 0.3 ml. on each of 3 consecutive days, repeated after 5 days with 0.2, 0.3, and 0.4 ml.; 12 to 14 days later all were bled in pools of 3 or 4. In another test, summarized in Table I, 10 RR and 10 SS mice were immunized in the same way except that the dosage of antigen was 0.2, 0.3, 0.4, and 0.3, 0.4, 0.4 ml.; these mice were tested individually. Controlled agglutination tests were made by means of the centrifuge method, or at 37° C. for 2 hours with fresh and heat-inactivated sera, employing the TAB vaccine undiluted (1,000 million typhoid, and 250 million each paratyphoid A and B heat-killed bacteria per milliliter) and equal amounts of twofold dilutions of the sera; also dilutions of antigen were tested as shown in Table I. The mixture was made either in 5 by 50 mm. tubes or in large-size capillary pipettes.

For the precipitation test, a similar technique for immunization was employed, except that undiluted horse serum, or alum-precipitated BPA, prepared by the method of Solotorovsky and Winsten,[5] was the antigen. Controlled precipitation reactions were read by means of the ring test, or by turbidity, in 5 by 50 mm. test tubes, or mainly in the capillary pipettes devised by Swift, Wilson, and Lancefield.[6] Either twofold dilutions of serum were tested against constant amounts of antigen or undiluted serum against twofold dilutions of antigen; equal quantity of both materials was used, with readings after 2 hours at 37° C. and overnight at 4° C. Table II summarizes one experiment in which 9 RR and 11 SS mice were immunized with BPA as described and which included a number

With the technical assistance of Sophie L. Skadorwa, A.B.
From the Laboratories of the Rockefeller Institute for Medical Research.
Received for publication June 26, 1954.

TABLE I. AGGLUTINATION OF TAB SUSPENSION BY ANTISERA DERIVING FROM INDIVIDUAL RESISTANT (RR) AND SUSCEPTIBLE (SS) MICE

| MOUSE BREED | SERUM NO. | POSITIVE IN RECIPROCAL OF DILUTION OF TAB | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2 | 10 | 20 | 40 | 80 | 160 | 320 |
| Normal stock | – | – | – | – | – | – | – | – |
| RR | 1 | + | + | + | + | + | – | – |
| | 2 | + | + | + | + | + | ± | ± |
| | 3 | + | + | + | + | + | + | – |
| | 4 | + | + | + | + | + | + | – |
| | 5 | + | + | + | + | ± | – | – |
| | 6 | + | + | + | + | ± | – | – |
| | 7 | + | + | + | + | + | – | – |
| | 8 | + | + | + | + | + | + | – |
| | 9 | + | + | + | + | + | + | – |
| | 10 | + | + | + | + | – | – | – |
| Normal stock | – | – | – | – | – | – | – | – |
| SS | 2 | + | + | + | + | + | + | – |
| | 3 | + | + | + | + | + | + | – |
| | 4 | + | + | + | + | + | + | – |
| | 5 | + | + | + | + | – | – | – |
| | 6 | + | + | + | + | + | + | + |
| | 7 | + | + | + | + | + | ± | – |
| | 8 | + | + | + | + | ± | ± | – |
| | 9 | + | + | + | + | + | – | – |

Capillary pipette test, 2 and 24 hours' reading; positive results at highest final dilution: ± = incomplete agglutination.

TABLE II. PRECIPITATION OF BOVINE PLASMA ALBUMIN BY ANTISERA DERIVING FROM RESISTANT (RR) AND SUSCEPTIBLE (SS) MICE

| MOUSE BREED | NEG. AT 1:2 | NO. OF MOUSE ANTISERA POSITIVE IN RECIPROCAL OF DILUTION* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1,024 | 2,048 |
| RR | 1 | 1 | 1 | 2 | 1 | | 1 | 1 | | | | 1 |
| SS | 1 | 3 | 1 | | 3 | 1 | | | 1 | | 1 | |
| Totals† | 2 | 4 | 2 | 2 | 4 | 1 | 1 | 1 | 1 | | 1 | 1 |

*Capillary pipette test, 2 and 24 hours' reading; positive results at highest final dilution.
†Totals, 1:2 to 1:16 dilution: RR 5; SS 7.
1:2 to 1:2,048 dilution: RR 8; SS 10.

TABLE III. SENSITIZATION OF MICE RESISTANT (RR) AND SUSCEPTIBLE (SS) TO DISSEMINATED ENCEPHALOMYELITIS WITH ALUM-PRECIPITATED BOVINE ALBUMIN (BPA)

| STOCK OF MICE | SEX | NO. SENSITIZED TO BPA* | CHALLENGE | | |
|---|---|---|---|---|---|
| | | | SIGNS | DEATHS IN 1 HR. | DEATHS % |
| SS | F | 12 | 12 | 10 | 69.6 |
| SS | M | 11 | 11 | 6 | |
| RR | F | 17 | 4 | 2 | 6.3 |
| RR | M | 15 | 1† | 0 | |

*BPA, 1 mg. total given subcutaneously in 4 interval doses, and challenged 4 weeks later with 1 mg. BPA introduced into tail vein.
†Only a slight, momentary reaction.

of nonimmunized controls. The mice were bled individually by cardiac puncture* and the test was carried out with constant amounts of serum mixed with twofold dilutions of BPA.

For testing anaphylaxis, the method for sensitization described by Solotorovsky and Winsten[5] was closely followed with the dosage of BPA which gave rise to 88 per cent

*All such operations were performed with the aid of deep ether anesthesia.

deaths after challenge; e.g., 0.25 ml. alum-precipitated BPA, 4 subcutaneous injections at 4-day intervals, with an intravenous shock dose after 4 weeks of 0.25 ml. (= 1 mg.) BPA.[5] So treated were 23 SS and 32 RR mice (Table III).

With respect to passive transfer to the two breeds of mice of antibody which might be revealed by anaphylaxis, it soon became apparent that this test would be beset by difficulties for want of an unequivocal assay method. In different experiments of orientation in untreated stock, W-Swiss mice: (a) 0.1 or 0.2 ml. of rabbit antibovine gamma globulin serum was introduced intraperitoneally and 24 hours later, 4 mg. of the globulin intravenously; (b) 4 mg. of the globulin was given first and 10 minutes later 0.3 ml. of the antiserum ("reverse passive" test); (c) rabbits were immunized with BPA[5] by means of 2 courses of 3 consecutive daily intraperitoneal injections of 1, 2, 3 and 2, 3, 4 mg. alum-precipitated BPA, and they were bled 14 days after the last dose. The antiserum precipitated BPA at ca. 1:16,000 dilution. For test in mice, 2 ml. of the antiserum was given intraperitoneally and 4 hours later 4 mg. of the BPA intravenously. Of the 3 tests, Tests a and b failed to show any definite signs of reaction, but in Test c one mouse died within an hour of anaphylaxis, another responded with characteristic signs but recovered, and 4 others, including controls, were unaffected. The latter test was now applied to 10 SS, 10 RR, and 9 stock W-Swiss mice; none of the 29 showed anaphylactic signs. Finally, the method of Munoz and associates[7] was applied to the problem, i.e., *H. pertussis* was injected before the introduction of antisera for the purpose of enhancing the anaphylactic response. In this instance, 4,500 million bacteria were given intraperitoneally to 15 RR, 15 SS, and 10 control stock W-Swiss mice. After 4 days 2 ml. rabbit antiserum (BPA precipitin titer, 1:32,000) was given intraperitoneally and 4 hours later 4 mg. BPA intravenously. All 10 controls and 15 mice each of the two breeds succumbed within 49 minutes after the shock dose was introduced. Thus it is shown here that passive transfer of antibody with a positive lethal anaphylactic reaction to a shock dose could be accomplished in 100 per cent of the mice. The significance of the results obtained in RR and SS mice was overshadowed, however, by the fact that this drastic test might have broken down any distinctive difference between the two. On the other hand, the causal agent of the active anaphylaxis test with the SS mice might not have been discernible by the procedure of passive transfer of the BPA antibody. Further work is therefore necessary to reduce the severity of the test, if it is possible, to a point where a satisfactory statistical result can be achieved.

For preparation of brain proteolipid A + B from both types of mice, the technique of Folch and Lees as carried out for cattle brain[8] was applied and adapted for mouse brain.[1] The tests of the encephalitogenic power of the mouse proteolipid combined with the Freund-type adjuvant have already been described in detail.[1, 2, 3, 9, 10]

## RESULTS

To summarize the results of the serologic tests: Agglutination, by means of the present procedures, of TAB suspensions occurred in dilutions up to 1:80 of the pooled serum of vaccinated mice, and to 1:320, at least, of the serum of such individual mice (Table I). No clear-cut difference was seen, however, in titer of the serum deriving from the RR and SS animals of both tests. So also for development of precipitation of horse serum: Reactions were noted in the serum of treated mice of both breeds in dilutions of 1:10 to 1:20; these ratios evidently reflect the limit of antibody dilution in such serum. However, when undiluted serum was tested against twofold dilutions of BPA it was found that positive precipitation could be observed in 1:2048 dilution. Again, no definite difference in titer could be noted in the serum obtained from the RR or the SS animals; Table II summarizes such a test.

While these serologic antibodies exhibited no clear-cut dissimilarity in the reactivity of the RR and SS mice after immunization, the results of the anaphylaxis tests yielded a wholly different picture as can be seen in Table III.

It will be noted from the tabulated results that of 23 SS and 32 RR mice all of the SS ones showed signs of anaphylactic shock and 16 died, and of the RR, only 5 exhibited signs and 2 died. The signs were similar to those already described[3] but, in most animals, more marked. After a few moments of quiet, the mice became excited with itchiness as reflected by biting and scratching, and ears and tails were cyanotic. Then scratching of nasal area supervened and dyspnea with wild movements, saltation, convulsive attacks, and death usually within 30 minutes. There is no explanation available at present on the somewhat milder reactions shown here by the males of both stocks. While the RR stock was generally 100 per cent resistant to ADE, the present test revealed a few reactors; the suggestion is offered that the assay for differentiation of the reactivity of RR from that of SS mice by development of disease is more delicate than by induction of hypersensitivity. However this may be, the results of the tests for anaphylaxis are in good agreement with the earlier ones[3] from which it was postulated that the basic mechanism of the susceptibility of SS mice to ADE is hypersensitivity.

The next series of tests concerned the presence of proteolipid A + B in the brain of the RR, or encephalomyelitis-resistant mice. With application of precisely similar technique to RR cerebral tissue, as was carried out with SS material, proteolipid A + B was recovered. This fraction, similar to that found in the brain of laboratory mixed strains of mice or in that of other species of animals,[3] was characteristically encephalitogenic. Thus, of 19 mice receiving this fraction, all exhibited ADE, most of them after the fourth or an even earlier injection. Differences in the expression of RR and SS mice, therefore, are not due to difference in the proteolipid as qualitatively determined here.

## DISCUSSION

It has been suggested[3] that the mechanism underlying the reaction of mice to acute disseminated encephalomyelitis following the inoculation of brain tissue plus adjuvant is based on hypersensitivity. The present results indicate that SS, or encephalomyelitis-susceptible mice, were readily sensitized to crystalline bovine plasma albumin, while the RR, or encephalomyelitis-resistant mice, were sensitized with difficulty or not at all, and thus lend support to this suggestion. On the other hand, both stocks of mice produced no clear-cut difference in titer of the serologic antibodies, agglutinins in the TAB-immunized, or of precipitins in the horse-serum- and BPA-immunized animals. Only in the test for anaphylaxis developed against bovine albumin was a difference noted in the two types of mice.

It is interesting in this relation that in 1925 Lewis and Loomis[11] demonstrated a differing susceptibility among inbred lines of guinea pigs to tuberculosis and those differing also varied in their response to an anaphylactic test; the most resistant were least responsive to the test. ADE is evidently not an infectious malady in the mouse[1] but the SS and RR animals are, respectively, susceptible and resistant to a bacterial disease, salmonellosis, and a virus infection, St. Louis encephalitis.[4, 12] Now, in the response of SS mice to the present anaphylactic test, ADE again resembles infectious experimental

tuberculosis in guinea pigs, the susceptibility of which is, in turn, governed by heritable factors[13] as are mice to experimental encephalomyelitis.[4] After the present experiments were concluded, Fink and Rothlauf[14] reported that among 5 inbred strains of mice sensitized to egg albumin, the variation in the ability of some of them to respond with anaphylactic shock was based on a genetic factor and that the reaction was not dependent on or correlated with circulating antibody.[15]

Finally, the mouse has been considered hitherto resistant to sensitization. As Raffel states it[16] this animal can be sensitized rather regularly with proper procedures. The results obtained here would support this statement which recently could also be affirmed by several investigators.[3, 5, 17]

## SUMMARY

Mice susceptible to anaphylactic sensitization with bovine plasma albumin were also susceptible to acute disseminated encephalomyelitis that is produced by the injection of normal brain tissue mixed with a Freund-type adjuvant. On the contrary, mice that are resistant to the development of encephalomyelitis were least responsive in such anaphylaxis tests. The susceptibility or resistance of the animals to development of both anaphylactic responses and encephalomyelitis was found not to be correlated with circulating antibodies of agglutinins for typhoid-paratyphoid A + B bacteria and of precipitins for horse serum and bovine plasma albumin.

## REFERENCES

1. Olitsky, P. K., and Yager, R. H.: Experimental Disseminated Encephalomyelitis in White Mice, J. Exper. Med. **90**: 213-224, 1949.
2. Olitsky, P. K., Casals, J., and Tal, C.: Relative Susceptibility of Various Stocks of Mice to Experimental Disseminated Encephalomyelitis, Proc. Soc. Exper. Biol. & Med. **75**: 276-279, 1950.
3. Olitsky, P. K., and Lee, J. M.: Biological Properties and Variations of Reactions of the Encephalitogenic Agent in Nervous Tissues, J. Immunol. **71**: 419-425, 1953.
4. Lee, J. M., Olitsky, P. K., Schneider, H. A., and Zinder, N. D.: Role of Heredity in Experimental Disseminated Encephalomyelitis in Mice, Proc. Soc. Exper. Biol. & Med. **85**: 430-432, 1954.
5. Solotorovsky, M., and Winsten, S.: Anaphylaxis in the Mouse Produced With Crystalline Bovine Albumin, J. Immunol. **71**: 296-299, 1953.
6. Swift, H. F., Wilson, A. T., and Lancefield, R. C.: Typing Group A Hemolytic Streptococci by M Precipitin Reactions in Capillary Pipettes, J. Exper. Med. **78**: 127-133, 1943.
7. Munoz, J., Schuchardt, L., and Verwey, W. F.: Passive Anaphylaxis in *H. pertussis* Treated Mice, Fed. Proc. **13**: 509, 1954.
8. Folch, J., and Lees, M.: Proteolipides, a New Type of Tissue Lipoproteins, J. Biol. Chem. **191**: 807-817, 1951.
9. Olitsky, P. K., and Tal, C.: Acute Disseminated Encephalomyelitis Produced in Mice by Brain Proteolipide (Folch-Lees), Proc. Soc. Exper. Biol. & Med. **79**: 50-53, 1952.
10. Tal, C., and Olitsky, P. K.: Quantitative Studies on Proteolipide as Incitant of Disseminated Encephalomyelitis in Mice, Science **116**: 420-421, 1952.
11. Lewis, P. A., and Loomis, D.: Allergic Irritability. II. Anaphylaxis in the Guinea Pig as Affected by the Inheritance, J. Exper. Med. **41**: 327-335, 1925.
12. Webster, L. T.: Inherited and Acquired Factors in Resistance to Infection. Development of Resistant and Susceptible Lines of Mice Through Selective Breeding, J. Exper. Med. **57**: 793-817, 1933.
13. Lewis, P. A., and Loomis, D.: Allergic Irritability. IV. The Capacity of Guinea Pigs to Produce Antibodies as Affected by the Inheritance and as Related to Familial Resistance to Tuberculosis, J. Exper. Med. **47**: 437-448, 1928.

14. Fink, M. A., and Rothlauf, M. V.: Variations in Sensitivity to Anaphylaxis and to Histamine in Inbred Strains of Mice, Proc. Soc. Exper. Biol. and Med. **85**: 336-338, 1954.
15. Fink, M. A., and Quinn, V. A.: Antibody Production in Inbred Strains of Mice, J. Immunol. **70**: 61-67, 1953. For additional important literature see Stern, K., and Davidsohn, I.: Heterohemoantibodies in Inbred Strains of Mice. I. Natural Agglutinins for Sheep and Chicken Red Cells, J. Immunol. **72**: 209-215, 1954; and Davidsohn, I., and Stern, K.: Ibid. II. Immune Agglutinins and Hemolysins for Sheep and Chicken Red Cells, pp. 216-223.
16. Raffel, S.: Immunity, Hypersensitivity, Serology, New York, 1953, Appleton-Century-Crofts, Inc., p. 211.
17. Weiser, R. S., Golub, O. J., and Hamre, D. M.: Studies on Anaphylaxis in the Mouse, J. Infect. Dis. **68**: 79-112, 1941; McMaster, P. D., and Kruse, H.: Peripheral Vascular Reactions in Anaphylaxis of the Mouse, J. Exper. Med. **89**: 583-596, 1949; Malkiel, S., Hargis, B. J., and Feinberg, S. M.: Anaphylactic Shock in the Mouse Vaccinated With *Hemophilus pertussis*. III. Antigens, Antibody and Passive Transfer Studies, J. Immunol. **71**: 311-318, 1953.

# THE EFFECT OF ANTIBIOTICS ON THE EXCRETION OF PHENOLIC COMPOUNDS

WALTER F. ROGERS, JR., M.D.,† MARY P. BURDICK, B.S., AND GEORGE R. BURNETT, M.D.
SYRACUSE, N. Y.

## INTRODUCTION

ABNORMALITIES in the metabolism of phenolic compounds have been described in pernicious anemia[1, 2] and scurvy.[3] In addition, phenolic compounds have been cited as substances that contribute to the clinical picture of uremia,[4, 5] and increased blood levels have been found in patients with liver disease.[6]

In the course of further investigations of tyrosine and phenol metabolism in man, we have observed that the oral administration of antibiotics was associated with a decreased excretion of certain phenolic compounds in the urine. This effect was limited chiefly to the fraction containing volatile phenols (*p*-cresol and phenol).

The pathologic alterations previously reported are not well understood and it is believed that the studies reported here will indicate the probable source of one group of urinary phenols and suggest a manner in which excretion of these may be influenced.

## METHODS

Five normal individuals and nine patients from a general medical service were studied before, during, and after the oral and parenteral administration of antibiotics. The urine was preserved with 1 to 2 ml. of concentrated sulfuric acid. In three patients, stools were stored under benzoic acid in four- to seven-day pools marked by carmine red. No attempt was made to regulate tyrosine and phenylalanine intake beyond that of routine diets. The antibiotics used were Aureomycin, Terramycin, chloramphenicol, and penicillin.* The phenolic content of the urines and stools was analyzed by the method of Shacter,**[7] with a few minor modifications.

This method consists of an acid hydrolysis of urine with sulfuric acid followed by continuous extraction with absolute ether. Thereafter the ether layer is extracted by hand, using sodium bicarbonate solution followed by extraction with sodium hydroxide. The amount of phenolic material present in each of the three fractions is determined by Millon's reaction. All samples were read against a standard of tyrosine so that each fraction is expressed as milligram equivalents of tyrosine.

---

From the Department of Medicine, University Hospital and Upstate Medical Center, State University of New York, Syracuse, N. Y.

This work was supported by a grant from the Hendricks Fund of the Upstate Medical Center, State University of New York.

Received for publication May 29, 1954.

†Deceased.

*The Aureomycin, Terramycin, chloramphenicol, and penicillin used were kindly supplied by Lederle Laboratories, C. Pfizer and Co., Parke, Davis & Co., and Bristol Laboratories, respectively.

**This method at present is unpublished. Personal correspondence with the author indicates it will be published in the near future. We are grateful to Dr. Shacter for his kind permission to use the method.

Pooled homogenized stool specimens were hydrolyzed with 7 N $H_2SO_4$. Aliquots were centrifuged and the supernatant layer was continuously extracted with ether for three hours. The stool extracts were then treated in the manner described for urine extracts.

Because of the presence of an amino group, compounds such as tyrosine, Tyramine, and epinephrine remain in the ether-insoluble fraction. Aromatic hydroxy acids (parahydroxyphenylacetic acid, parahydroxyphenylpyruvic acid, parahydroxyphenylpropionic acid, and parahydroxybenzoic acid) are soluble in ether and extractable by weak bases such as sodium bicarbonate. Volatile phenols like phenol and *p*-cresol are soluble in ether but require a strong base such as sodium hydroxide for extraction.[7]

Polyhydric phenols such as resorcinol, phloroglucinol, catechol, and orcinol are also found in this last fraction. However, these compounds do not give a Millon reaction and are consequently not measured.

## RESULTS

Table I indicates that when a representative volatile phenol is added to urine, its recovery in the NaOH fractions is satisfactory.

Table II shows that the antibiotics used in this study have no effect on previously determined phenol values when they are added directly to urine and fractionation is then performed.

TABLE I. RECOVERY OF PHENOL ADDED TO URINE (NaOH SOLUBLE OR VOLATILE PHENOL FRACTION)

| URINE SAMPLE | PHENOL ADDED (MG.) | PHENOL RECOVERED (MG.) | PER CENT RECOVERY |
|---|---|---|---|
| 1 | 110 | 100 | 91 |
| 2 | 104 | 95 | 91.5 |
| 3 | 111 | 110 | 99 |

TABLE II. EFFECT OF THE DIRECT ADDITION OF ANTIBIOTICS TO URINE ON PREVIOUSLY DETERMINED URINARY PHENOL VALUES

| URINE NUMBER | URINARY FRACTIONS | URINE UNTREATED MG./24 HR. AS TYROSINE | URINE PLUS ANTIBIOTICS | |
|---|---|---|---|---|
| | | | MG./24 HR. AS TYROSINE | CONCENTRATION OF ANTIBIOTIC |
| 1 | Ether insoluble | 100 | 108 | 1 mg. |
| | $NaHCO_3$* | 130 | 140 | Terramycin |
| | NaOH† | 190 | 195 | per ml. |
| 2 | Ether insoluble | 108 | 120 | 1 mg. |
| | $NaHCO_3$ | 180 | 178 | Aureomycin |
| | NaOH | 189 | 180 | per ml. |
| 3 | Ether insoluble | 93 | 95 | 1 mg. |
| | $NaHCO_3$ | 224 | 234 | chloramphenicol |
| | NaOH | 115 | 112 | per ml. |
| 4 | Ether insoluble | 98 | 94 | 1,500 units |
| | $NaHCO_3$ | 246 | 233 | penicillin |
| | NaOH | 89 | 95 | per ml. |

*$NaHCO_3$ refers to compounds soluble in ether but extracted with sodium bicarbonate solution, i.e., aromatic hydroxy acids.

†NaOH refers to compounds soluble in ether but extracted with sodium hydroxide solution, i.e., volatile phenols.

*Effect of Oral Terramycin, Aureomycin, and Chloramphenicol.*—The data indicate that all of these antibiotics depress the urinary excretion of "volatile phenols."

Fig. 1 shows the data on the patient in whom this phenomenon was first noted. He was a 65-year-old man with untreated pernicious anemia who developed pneumonia and was treated with 2 Gm. of Terramycin daily. Unfortunately, pretreatment values were not available, but it is clear that on the third day of therapy the volatile phenol fraction of the urine had diminished to about 40 per cent of previous values and that on the fourth day no phenols were detectable in this fraction. This effect persisted during the remaining twelve days while the patient was continued on 2 Gm. of Terramycin daily, meanwhile receiving liver extract therapy. There was no change in the other two phenolic fractions.

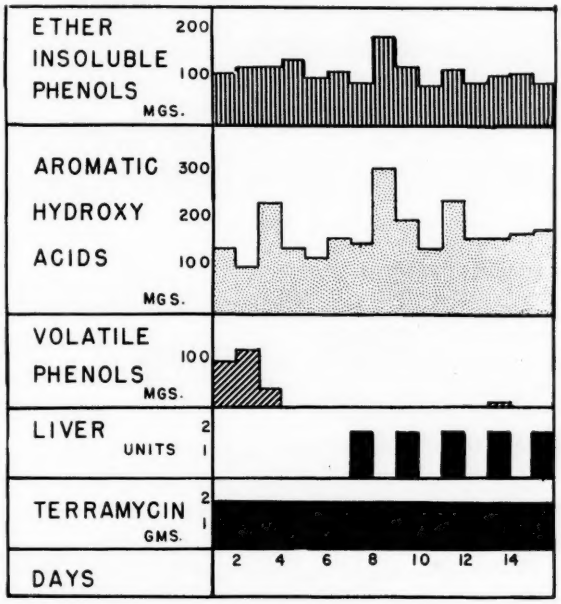



Fig. 1.—Effect of oral Terramycin on urinary phenol excretion. A. E.—Convalescent pneumonia and pernicious anemia.

The effect of Terramycin on the excretion of phenols in a normal 29-year-old man is shown in Fig. 2. After a three-day control period he was given 2 Gm. of Terramycin daily for five days. This dosage resulted in a prompt fall in the urinary volatile phenol fraction accompanied by a suggestive fall in the aromatic hydroxy acid (sodium bicarbonate) fraction. For the next five-day period he received no antibiotic, yet the volatile phenol excretion remained depressed. At the time of this experiment, it was not appreciated that a considerable period of time was necessary for phenol metabolism to return to normal following the ingestion of antibiotics, and the patient was given 1 Gm. of Terramycin for five days before pretreatment levels had been regained. This second course of drug had no depressing effect although it may have prevented a further rise. After cessation of Terramycin, eleven days elapsed before the volatile phenol fraction returned to control levels. During this period there was also a considerable rise in the aromatic hydroxy acid fraction.

Fecal as well as urinary phenols were studied in three other patients receiving Terramycin. In two patients results were similar and the data on one of these patients, a 40-year-old man with cirrhosis, are presented in Fig. 3. After a control period there was a fall in the urinary excretion of volatile phenols during the daily administration of 2 Gm. of Terramycin by mouth.

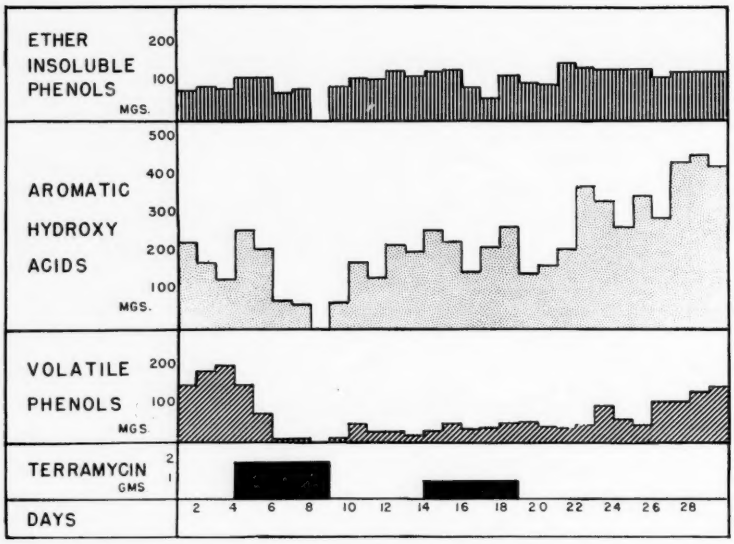



Fig. 2.—Effect of oral Terramycin on urinary phenol excretion. R. L.—Normal male.

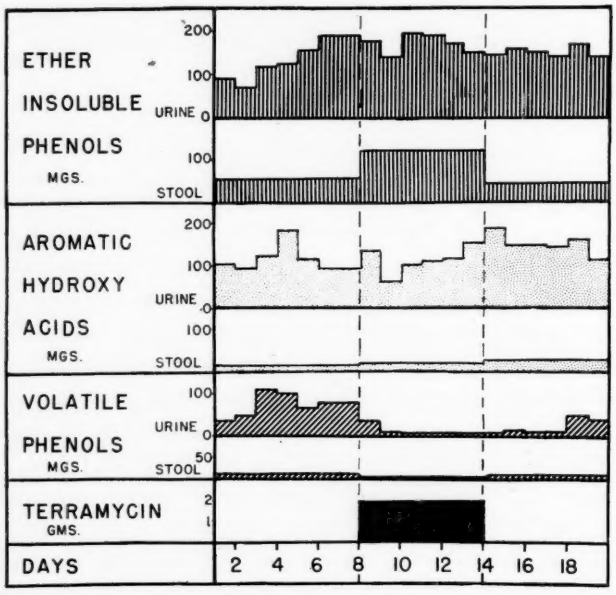



Fig. 3.—Effect of oral Terramycin on urine and stool phenols. R. R.—Hepatic cirrhosis.

Significant change in the other urinary phenolic fractions did not occur. In the postantibiotic period there was a gradual return toward pretreatment values. Analysis of the stool phenols indicated that during Terramycin administration ether insoluble phenolic compounds rose to more than twice control levels, while the volatile phenol fraction fell to 10 per cent of pretreatment levels. No significant change occurred in the aromatic hydroxy acid fraction. In the posttreatment period, fecal phenols returned toward control levels.

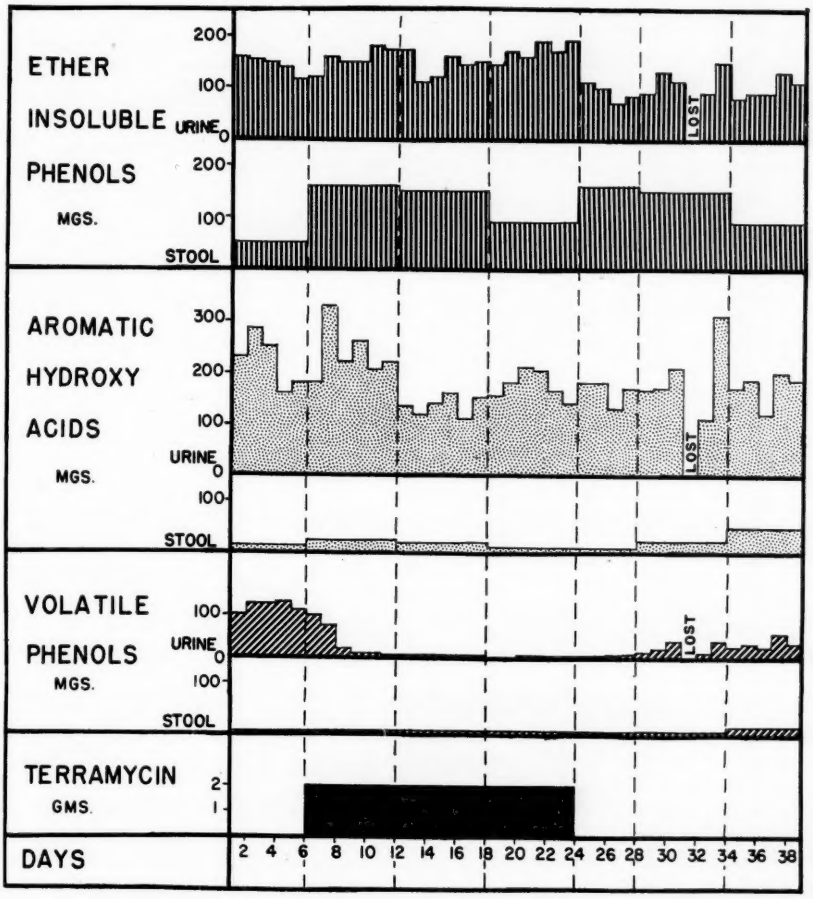



Fig. 4.—Effect of oral Terramycin on urine and stool phenols. F. H.—Hepatic cirrhosis.

In a third patient (Fig. 4), results were comparable except that the initial level of fecal volatile phenols was so low that a fall did not take place when Terramycin was administered. A rise in this fraction did occur, however, after Terramycin was stopped. This patient was convalescing from pneumonia and had received parenteral penicillin until three days before this study began.

In all three patients in whom fecal phenols were determined it was noted that the excretion of all phenolic compounds was predominantly renal as compared to the intestinal tract.

TABLE III. EXCRETION OF THE NaOH FRACTION (VOLATILE PHENOLS) IN MILLIGRAMS PER DAY

| | CHLORAMPHENICOL | | AUREOMYCIN | | ORAL PENICILLIN | | | PARENTERAL PENICILLIN |
|---|---|---|---|---|---|---|---|---|
| DOSE: | 2 GM./DAY | 1.5 GM./DAY | 2 GM./DAY | 1 GM./DAY | 1.5 MILLION UNITS/DAY | 1.5 MILLION UNITS/DAY | 1 MILLION UNITS/DAY | 1.5 MILLION UNITS/DAY |
| PATIENT: | E. C. | J. S. | F. F. | A. N. | M. S. | C. T. | R. S. | E. P. |
| DIAGNOSIS: | Senile osteoporosis | Pulmonary tuberculosis | Gastritis | Duodenal ulcer | Normal | Normal | Normal | Cirrhosis |
| *Day* | *Mg.* | *Mg.* | *Mg.* | *Mg.* | *Mg.* | *Mg.* | *Mg.* | *Mg.* |
| 1 | 87 | 90 | 80 | 40 | 70 | 90 | 114 | 60 |
| 2 | 90 | 110 | 140 | 50 | *50 | *22 | 120 | 65 |
| 3 | 82 | 70 | 90 | *30 | *20 | *19 | *93 | 75 |
| 4 | *60 | *50 | 130 | *35 | *10 | *5 | *23 | *50 |
| 5 | *30 | *30 | 100 | *10 | | | *23 | *30 |
| 6 | *20 | *25 | *50 | *20 | | | *44 | |
| 7 | *10 | *24 | *60 | *15 | | | *56 | *35 |
| 8 | *11 | *27 | *42 | 10 | | | 76 | *30 |
| 9 | 10 | *25 | *51 | 15 | | | 69 | *33 |
| 10 | 10 | *20 | *55 | 15 | | | 84 | 30 |
| 11 | 20 | | *49 | 30 | | | | 35 |
| 12 | 30 | | *25 | 40 | | | 78 | |
| 13 | 30 | | *40 | 75 | | | 99 | |
| 14 | 35 | | *55 | | | | | 45 |
| 15 | -- | | 82 | | | | | 50 |
| 16 | -- | | 130 | | | | | |
| 17 | -- | | | | | | | |
| 18 | 45 | | | | | | | |
| 19 | 60 | | | | | | | |
| 20 | 65 | | | | | | | |
| 21 | 80 | | | | | | | |

*Indicates days that patient received antibiotic.

Studies were also carried out in patients receiving Aureomycin and chloramphenicol. The results of these studies are given in Table III. In general, the data are comparable to those obtained with Terramycin. However, in the dosage used, Terramycin was the most effective agent in causing a decrease in volatile phenol excretion.

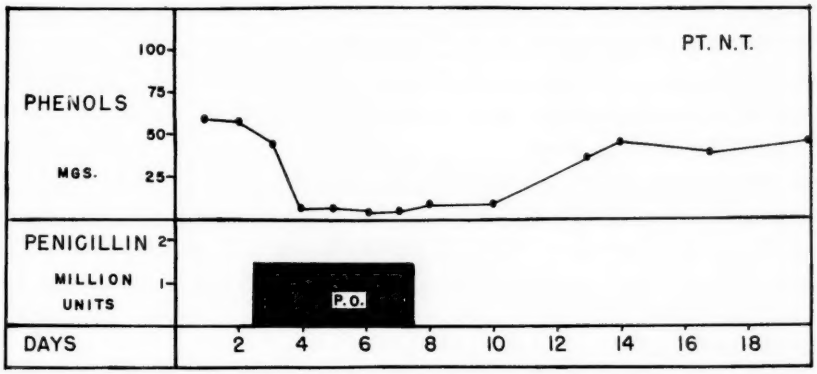



Fig. 5.—Effect on urinary volatile phenols of 1.5 million units of penicillin by mouth for five days. N. T.—Normal male.

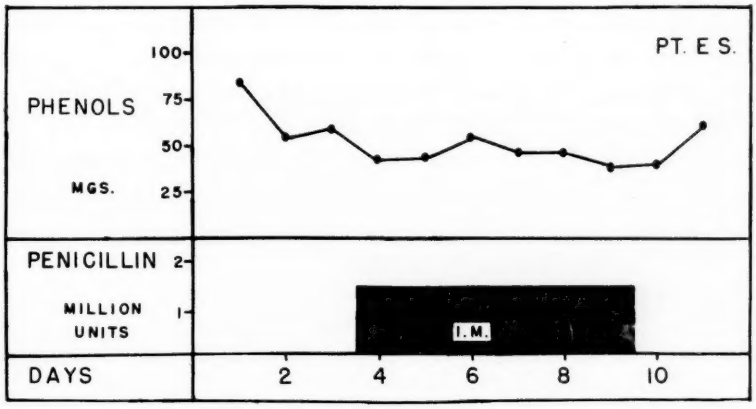



Fig. 6.—Effect on urinary volatile phenols of 1.5 million units of penicillin, intramuscularly for six days. R. S.—Normal male.

*Effect of Oral and Parenteral Penicillin.*—When given in sufficient doses, oral penicillin also depresses the urinary excretion of volatile phenols. Fig. 5 shows the effect of 1.5 million units of penicillin given orally to a normal man. The volatile phenols dropped quickly, remained low as long as penicillin was continued, and slowly rose to control levels seven days after penicillin had been stopped. The data on three additional patients receiving oral penicillin are given in Table III.

The effect of parenterally administered penicillin upon volatile phenol excretion was studied in two individuals (Fig. 6, Table III). Subject R. S., a normal male, was given 1.5 million units intramuscularly daily for six days, and

patient E. P. with hepatic cirrhosis received the same dose for five days. A decrease in the excretion of these compounds occurred during the intramuscular injection of penicillin but this was less marked than the effect obtained with oral administration of this antibiotic.

## DISCUSSION

The data presented indicate that the oral administration of Terramycin, Aureomycin, chloramphenicol, and penicillin have a marked effect on the urinary excretion of one group of phenols. This fraction of phenols is soluble in ether, requires a strong base for extraction from ether, and is composed chiefly of phenol and *p*-cresol.

The significance of these findings with respect to tyrosine and phenol metabolism can be outlined as follows. It is probable that the major source of phenolic compounds in the body is tyrosine, which is derived from ingested protein and synthesized from phenylalanine. Studies on normal patients and on patients with abnormalities of tyrosine metabolism (E. G., alkaptonuria,[8] tyrosinosis,[9] and premature infants fed high-protein diets[10]) have indicated that the metabolism of tyrosine proceeds by the formation of *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid, 2,5-dihydroxyphenylpyruvic acid and homogentisic acid in that order. After the formation of homogentisic acid the aromatic ring is opened to form fumarylacetoacetic acid and the phenolic structure no longer exists. In the urine, however, there are a number of other phenolic compounds, namely, *p*-hydroxyphenylpropionic acid, *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid, *p*-cresol, and phenol, which are not accounted for by the above outlined systemic metabolism of tyrosine. These remaining phenols are thought to originate in the gastrointestinal tract as the result of bacterial action on tyrosine.[11, 12] Subsequently, an unknown proportion of these is absorbed, conjugated by the liver, and excreted in the urine.

The data presented above indicate that when antibiotics are given by mouth the fraction of urinary phenols which contains phenol and *p*-cresol either disappears or decreases to negligible levels and that there is no marked change in the other fractions of urinary phenols. However, in one patient receiving Terramycin, R. L. (Fig. 2), there was a suggestive fall in the aromatic hydroxy acid fraction. This change was not constant in all patients and requires further study. Concomitant fecal studies show that the excretion of phenol and *p*-cresol by this route also decreases, but at the same time the fecal excretion of tyrosine increases.

These findings are thus consistent with the concept that antibiotics given orally change stool flora or bacterial metabolism and thereby decrease the production of phenol and *p*-cresol from tyrosine in the intestine. This leads to less phenols being available for absorption and hence decreased amounts of these substances are excreted in the urine. It cannot be said that this is necessarily true of *p*-hydroxyphenylpropionic acid, *p*-hydroxyphenylacetic acid, or *p*-hydroxybenzoic acid because the urinary and stool phenolic fractions containing these compounds did not change greatly during antibiotic therapy.

Volatile phenol excretion began to fall within 24 to 48 hours and reached a minimum by 72 to 96 hours. The longest period of antibiotic (Terramycin) administration in this study was 18 days and volatile phenol excretion remained less than 5 mg. per day during this time. After an effective suppressing dose has been given, considerable time (10 to 15 days) is required for excretion of volatile phenols to return to normal.

At the present time, it is not possible to say whether this depression of the formation and excretion of volatile phenols by antibiotics is of clinical interest. However, as stated previously, phenols have been cited as contributing to the clinical picture of uremia[4, 5] and have been found to be elevated in liver disease.[6] Antibiotics have also been found to prolong life in experimental liver disease.[13] If the production of volatile phenols in the intestine can be suppressed by antibiotics, and lesser quantities are therefore available for absorption as suggested by the small amounts appearing in the urine, it is perhaps possible that the blood stream can be cleared of these substances.

## SUMMARY

Data have been presented to show that when sufficient doses of Terramycin, Aureomycin, chloramphenicol, or penicillin are given by mouth, the urinary excretion of volatile phenolic compounds (principally phenol and *p*-cresol) decreases markedly. Parenteral penicillin had only a small and perhaps negligible effect on the urinary excretion of these phenols. In the case of Terramycin, fecal phenols were also measured and volatile phenol compounds were found to be decreased whereas the fraction containing their probable precursor, tyrosine, rose during antibiotic administration.

These findings appear to be consistent with the concept that volatile phenols are formed from tyrosine in the gastrointestinal tract by the action of bacteria. Disturbances in the number, type, and metabolism of bacteria as a result of orally administered antibiotics could result in decreased tyrosine conversion to volatile phenols with diminished absorption and hence decreased excretion.

## REFERENCES

1. Swendseid, M. E., Wandruff, B., and Bethell, F. H.: Urinary Phenols in Pernicious Anemia, J. LAB. & CLIN. MED. **32**: 1242, 1947.
2. Abbott, L. D., Jr., and James, G. W., III: Effect of Vitamin $B_{12}$ on the Urinary Phenol Fractions in Pernicious Anemia, J. LAB. & CLIN. MED. **35**: 35, 1950.
3. Rogers, W. F., and Gardner, F. H.: Tyrosine Metabolism in Human Scurvy, J. LAB. & CLIN. MED. **34**: 1491, 1949.
4. Harrison, T. R., and Mason, M. F.: The Pathogenesis of the Uremic Syndrome, Medicine **16**: 1, 1937.
5. Nesbit, R. M., Buck, L., and Olsen, M. S.: Level of Blood Phenol in Uremia, Arch. Surg. **53**: 483, 1946.
6. Murphy, T. L., Chalmers, J. C., Eckhardt, R. D., and Davidson, C. S.: Hepatic Coma, New England J. Med. **239**: 605, 1948.
7. Shacter, B.: A Study of the Behavior of the Aromatic Amino Acids, Tyrosine and Phenylalanine. Dissertation for the degree of Doctor of Philosophy in Biochemistry, University of Michigan, 1943.
8. Galdston, M., Steele, J. M., and Dobriner, K.: Alcaptonuria and Ochronosis, Am. J. Med. **13**: 432, 1952.

9. Medes, G.: A New Error of Tyrosine Metabolism, Tyrosinosis: The Intermediary Metabolism of Tyrosine and Phenylalanine, Biochem. J. 26: 917, 1932.
10. Levine, S. Z., Marples, E., and Gordon, H. H.: A Defect in the Metabolism of Tyrosine and Phenylalanine in Premature Infants. I. Identification and Assay of Intermediary Products, J. Clin. Invest. 20: 199, 1941.
11. Folin, O., and Denis, W.: The Excretion of Free and Conjugated Phenols and Phenol Derivatives, J. Biol. Chem. 22: 309, 1915.
12. Pelham, K. F., and Whipple, G. H.: The Physiology of Phenols, J. Biol. Chem. 50: 499, 1922.
13. György, P., Stokes, J., Jr., Smith, W. H., and Goldblatt, H.: Studies on the Use of Aureomycin in Hepatic Disease. II. The Effect of Aureomycin on Experimental Dietary Hepatic Necrosis, Am. J. M. Sc. 220: 6, 1950.

# IN VITRO ACTIVITY OF CHLORAMPHENICOL ON SALMONELLA TYPHI

KENNETH C. WATSON,* M.B., CH.B. (ABER.)
PIETERMARITZBURG, SOUTH AFRICA

DURING the investigation of 114 cases of typhoid fever, all of which were treated with chloramphenicol, *S. typhi* was isolated from blood clot in twenty of these during the time that the patients were taking the antibiotic. Details of some of these have already been considered.[1] In one instance a positive isolation was obtained after eleven days of treatment (total dosage 22 Gm.), in another after ten days (total dosage 10 Gm.), and in a third after eight days (total dosage 8 Gm.). In every case chloramphenicol was given in doses of either 250 mg. or 500 mg. every six hours, depending on the severity of the case. The method of clot culture employed was that utilizing streptokinase bile salt broth as the culture medium.[2] The streptokinase produces rapid clot lysis and releases any trapped organisms into the medium. Blood levels of chloramphenicol in each of the above twenty cases were found to lie between 15 and 40 μg per milliliter at the time when positive blood samples were obtained.

In view of these findings an *in vitro* study of the effect of chloramphenicol on *S. typhi* was undertaken.

## METHODS AND RESULTS

A. *Effect of Chloramphenicol on S. typhi in Artificially Infected Clots.*—

1. *Procedure:* Three rows of tubes containing ten tubes per row were set up and 0.5 ml. amounts of a saline suspension of *S. typhi* were added to each. The suspension was prepared from an overnight digest broth culture and each 0.5 ml. contained approximately 100 viable organisms, as estimated by plate count. Five-tenths milliliter amounts of chloramphenicol solution were added to each tube of Row 1, to reach a final concentration of 50 μg per milliliter, and to each tube of Row 2, to reach a final concentration of 25 μg per milliliter; 0.5 ml. of saline was added to each tube of Row 3. After ten minutes at room temperature 3.0 ml. of blood from a donor was added to each tube and allowed to clot after the contents were mixed. The tubes were then incubated at 37.0° C. for four hours and the separated serum was removed. Each clot was then added to 15 ml. of streptokinase bile salt broth in a universal container and incubated at 37.0° C. for seven days. Subcultures were plated daily onto Wilson and Blair's medium.

The above procedure was repeated in the same way, except that chloramphenicol and bacterial suspension were allowed to interact for varying periods of time before the blood was added, viz., 4, 24, and 48 hours. Results of subculture are detailed in Table I.

2. *Results:* In spite of the interaction of chloramphenicol and bacterial suspension for four hours at room temperature at a concentration of 50 μg per milliliter, positive isolations were obtained from 100 per cent of the blood

Received for publication July 28, 1954.

*Assistant Pathologist, Natal Provincial Administration, Grey's Hospital, Pietermaritzburg, South Africa.

clots, and from 80 per cent after twenty-four hours. After forty-eight hours interaction of chloramphenicol and bacterial suspension, the number of positive isolations amounted to only 10 per cent at 50 μg per milliliter, and 20 per cent at 25 μg per milliliter. With both concentrations the maximum number of positive isolations was delayed, compared to the control tubes, following four, twenty-four, and forty-eight hours interaction of chloramphenicol and suspension. These findings suggest a slow bactericidal action of chloramphenicol on *S. typhi.*

TABLE I. EFFECT OF CHLORAMPHENICOL ON *S. typhi* IN ARTIFICIALLY INFECTED CLOTS

| TIME OF INTERACTION OF CHLORAMPHENICOL AND BACTERIAL SUSPENSION | 10 MINUTES | 4 HOURS | 24 HOURS | 48 HOURS |
|---|---|---|---|---|
| Per cent positive isolations | | | | |
| 50 μg/ml. | 100 | 100 | 80 | 10 |
| 25 μg/ml. | 100 | 100 | 90 | 20 |
| 0 μg/ml. | 100 | 100 | 100 | 100 |
| Number of days incubation required for maximum number of positive isolations | | | | |
| 50 μg/ml. | 2 | 4 | 5 | 3 |
| 25 μg/ml. | 1 | 3 | 4 | 4 |
| 0 μg/ml. | 1 | 1 | 1 | 1 |

B. *Growth Study of S. typhi in the Presence of Chloramphenicol.*—

1. *Procedure:* Two concentrations of chloramphenicol, 200 μg per milliliter and 25 μg per milliliter were prepared in 10 ml. amounts of digest broth, in sterile five by five-eighths inch tubes. One-tenth milliliter of a $\frac{1}{10,000}$ saline dilution of an overnight broth culture of a freshly isolated strain of *S. typhi* was added to each. A control tube without chloramphenicol was included. At varying intervals of incubation at 37.0° C., 0.1 ml. amounts were removed and added to 0.9 ml. amounts of sterile 0.85 per cent saline. Serial tenfold dilutions were prepared and plate colony counts of viable organisms performed. Results are shown in Fig. 1.

2. *Results:* With a concentration of 25 μg per milliliter no bactericidal effect was evident during the first twelve hours of the growth study. Between twelve and twenty-four hours there was a slow but gradual decline in the bacterial population, but even after forty-eight hours viable organisms were still present. At a concentration of 200 μg per milliliter the bactericidal effect was more rapid, though even with this high concentration only a bacteriostatic action was evident during the first eight hours.

These findings are to some extent in agreement with those of Bliss and Todd,[3] regarding the action of chloramphenicol on *E. coli.* They, however, found the bactericidal action to be more rapid than that shown above, a concentration of 10 μg per milliliter producing a bacteriostatic action only during the first five hours of incubation. The authors conclude, "Chloromycetin behaves like Aureomycin during the early stages of a growth study, but the decline in bacterial population is maintained and by 24 or 48 hours no viable organisms are found." Bliss and Todd[3] found that the inoculum size did not affect the minimal inhibitory concentrations of chloramphenicol as much as it did that

of other antibiotics. This is our experience also, curves of similar form to those in Fig. 1 being obtained when the growth study was repeated with 1/100,000 and 1/1,000,000 dilutions of bacterial culture.

## DISCUSSION

Conflicting reports are to be found in the literature concerning the bactericidal or bacteriostatic action of chloramphenicol. Smadel,[4] for example, states, "Chloramphenicol in concentrations of a few gamma per c.c. in culture media will inhibit the growth of *S. typhi,* but even a thousand gamma will not kill the organisms." Magoffin and Spink[5] also demonstrated a primarily bacteriostatic action for chloramphenicol, and Matteucci and associates[6] stress the suppressive rather than the curative action of the antibiotic. The work of Bliss and Todd,[3] however, suggests that chloramphenicol has a slow but definite, bactericidal effect, and Hobby and co-workers[7] express similar opinions. The results shown above would appear to support the findings of these latter workers.

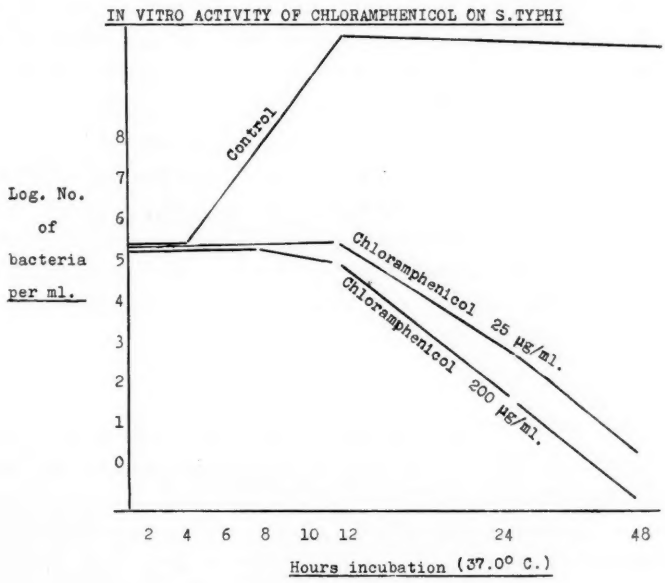



Fig. 1.

If the action of chloramphenicol in vivo was merely a bacteriostatic one, then a rational explanation for the occurrence of the twenty positive isolations from blood clot, during the exhibition of chloramphenicol therapy, would be forthcoming. In this event removal of the serum containing the chloramphenicol would be sufficient. The suppressed organisms in the residual clot would then be allowed to grow. If, however, the slow bactericidal action demonstrable in vitro, also takes place in vivo, then in this event complete sterility of the peripheral blood would be expected in twenty-four to forty-eight hours, de-

pending on the blood levels of the antibiotic. This, as has been shown, does not always occur, suggesting that viable organisms may in some way be protected from the action of a circulating antibiotic.

Goodpasture[8] and Adams[9] have both reported the presence of intracellular forms of *S. typhi* in intestinal lymphoid follicles and in mesenteric lesions. Magoffin and Spink[5] have shown intracellular protection of forms of *Brucella abortus,* phagocytosed by cells of the buffy coat, from the action of streptomycin. We have demonstrated similar protection in the case of *S. typhi* and have advanced the hypothesis that *S. typhi* may be phagocytosed in vivo and be protected from the action of chloramphenicol.[1] It would seem that such intracellular forms may remain viable and be released periodically from the cells giving rise to a transient bacteremia. In view of the slow bactericidal action of chloramphenicol, blood sampled during these periods will give a positive isolation if the chloramphenicol is removed in the serum or diluted by an adequate volume of medium. Reilly and associates[10] and Marmion[11] have recovered *S. typhi* from various organs post mortem, in spite of chloramphenicol therapy during life, and it seems probable that this represents recovery of such intracellular forms. Regarding the hypothesis that intracellular forms are released in a viable state, some support is to be found in the work of Adams[9] and, more recently, of Wilson.[12] The former states, "In occasional cells containing large colonies, the vacuoles in which they lie appear to have ruptured and the bacilli appear to be exuding into the adjacent tissue." Wilson has demonstrated by phase contrast microscopy that organisms, e.g., streptococci, may be ingested by leukocytes and subsequently egested in a still viable form. It also seems probable that intracellular collections of organisms may be rationally invoked to explain the occurrence of relapses in cases of typhoid fever treated with chloramphenicol.

## SUMMARY

The occurrence of positive clot cultures in cases of typhoid fever on treatment with chloramphenicol led to an in vitro study of the action of the antibiotic on *S. typhi.* Chloramphenicol appears to have a slow but definite bactericidal action on *S. typhi.* A possible correlation between the bactericidal action and the occurrence of positive cultures is discussed.

I am indebted to Dr. J. C. Thomas, Provincial Pathologist, Natal Provincial Administration, for his continued interest in this work.

## REFERENCES

1. Watson, K. C.: Effect of Chloramphenicol on the Isolation of *S. typhi* From the Blood Stream, Brit. J. Clin. Path.
2. Thomas, J. C., Watson, K. C., and Hewstone, A. S.: The Use of Streptokinase Bile Salt Broth for Clot Cultures in the Diagnosis of Enteric Fever, Brit. J. Clin. Path. 7: 50, 1954.
3. Bliss, E. A., and Todd, H. P.: A Comparison of Eight Antibiotic Agents *in vivo* and *in vitro,* J. Bact. 58: 61, 1949.
4. Smadel, J. E.: Chloramphenicol (Chloromycetin) and Tropical Medicine, Tr. Roy. Soc. Trop. Med. & Hyg. 43: 555, 1950.

5. Magoffin, R. L., and Spink, W. W.: The Protection of Intracellular Brucella Against Streptomycin Alone and in Combination With Other Antibiotics, J. LAB. & CLIN. MED. **37**: 924, 1951.
6. Matteucci, W. V., Schimmel, H. N., and Boger, W. P.: Typhoid Fever: Chloramphenicol Therapy and Problem of Relapse, Am. J. M. Sc. **222**: 446, 1951.
7. Hobby, G. L., Lenert, T. F., and Doughtery, N.: Newer Antibiotics: Factors Influencing Their Antimicrobial Activity, Ann. New York Acad. Sc. **53**: 27, 1950.
8. Goodpasture, E. W.: Confirming Pathogenesis of Typhoid Fever, Am. J. Path. **13**: 175, 1937.
9. Adams, J. W.: Intracellular Bacilli in Intestinal and Mesenteric Lesions of Typhoid Fever, Am. J. Path. **15**: 561, 1939.
10. Reilly, J., Compagnon, A., Tournier, P., Bastin, R., and Du Buit, H.: Les accidents du traitment des fievres typhoides par la Chloromycetine. Etude experimentale et deductions therapeutiques, Ann. méd. **51**: 597, 1950.
11. Marmion, D. E.: The Treatment of Typhoid Fever With Chloramphenicol: A Clinical Study of 330 Cases of Enteric Fever Treated in Egypt, Tr. Roy. Soc. Trop. Med. & Hyg. **46**: 619, 1952.
12. Wilson, A. T.: The Egestion of Phagocytosed Particles by Leucocytes, J. Exper. Med. **98**: 305, 1953.

# EFFECT OF ACUTE RENAL FAILURE ON THE PLASMA VOLUME AND VOLUME OF PACKED RED CELLS IN THE DOG

HAROLD BROWN, M.D., A. A. ILIYA, M.D., P. B. PRICE, M.D., AND O. N. RAMBO, JR., M.D.
SALT LAKE CITY, UTAH

## INTRODUCTION

IN RECENT years considerable attention has been devoted to the problem of therapy in acute renal failure. Many writers have pointed out the tendency of patients with acute renal failure to develop congestive failure and pulmonary edema.[1-6] These complications have been thought to result largely from overzealous treatment. It, therefore, seemed important to study the changes in plasma volume, volume of packed red cells (hematocrit) and electrolytes in anuric animals which were given no treatment which might disguise of accentuate the changes.

## MATERIALS AND METHODS

Eighteen mongrel dogs, obtained from the local pound, were studied. Several weeks prior to the experiment, the animals were splenectomized in order to stabilize the plasma volume determinations which are difficult to reproduce in dogs with intact spleens. After recovery from the procedure, the base-line set of determinations was made. Included were the estimation of the volume of packed red blood cells (hematocrit) with the Wintrobe tube, the plasma volume by the method of Price and Longmire[7] using multiple injections of Evans blue dye, the plasma levels of sodium and potassium by the flame photometer, chloride,[8] inorganic phosphorus,[9] urea nitrogen,[10] and creatinine.[11] In eight animals, plasma volume was measured also by means of radioactive iodine-tagged human serum albumin[12] to confirm the results obtained with the Evans blue dye technique. Postoperatively, the determinations listed above were repeated daily until the animals expired.

The dogs were anesthetized with intravenous pentobarbital (35 mg. per kilogram) and the ureters were tied through a low midline incision. Postoperatively, twelve of the animals were starved and six were permitted access to food and water. Since the animals allowed food and water vomited much of it and since the changes in plasma volume and hematocrit in the two groups were not significantly different, these data have been analyzed together.

Gross and microscopic pathologic examination was performed post mortem on five of the animals.

## RESULTS

Quantitative results are listed in Table I.

A. *Changes in Plasma Volume.*—A scattergram (Fig. 1) indicates the per cent changes in plasma volume of each animal on the days following surgery. It can be seen that considerable variation occurred from one animal to another. However, the mean plasma volume rose steadily and reached a peak on the third postoperative day. At this time the mean plasma volume of the twelve

From the Veterans Hospital and the Departments of Medicine, Surgery and Pathology, University of Utah College of Medicine, Salt Lake City, Utah.
Received for publication July 12, 1954.

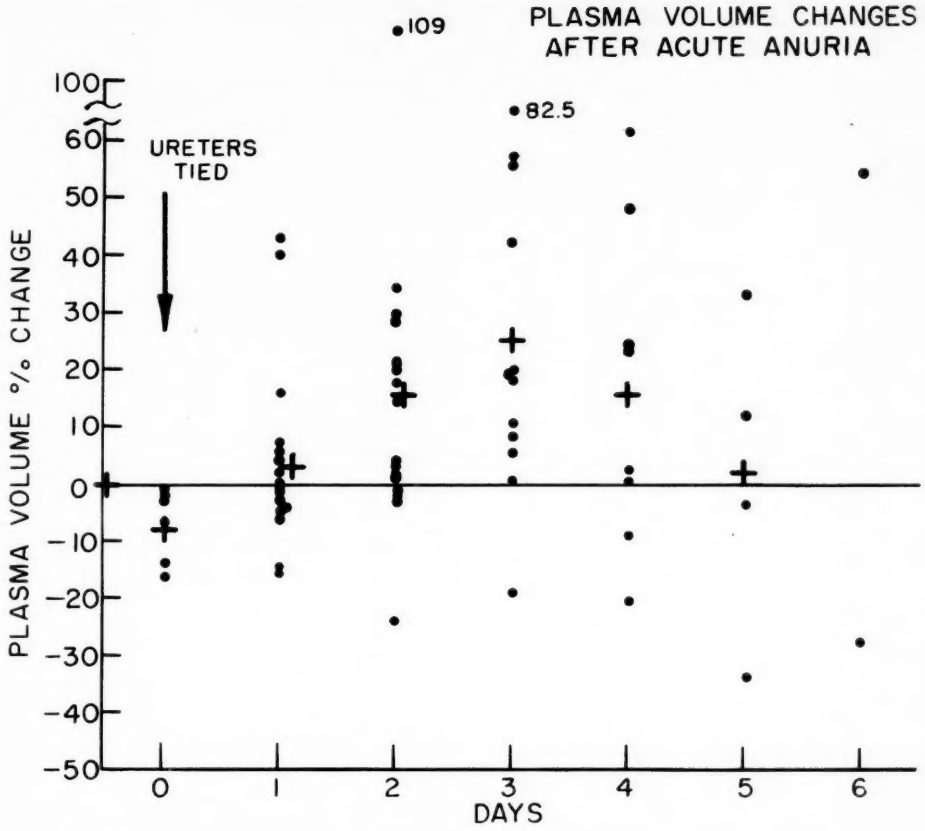



Fig. 1.—Scattergram showing the per cent change in plasma volume of each animal on the days following ureteral ligation. The day of ureteral ligation is designated as day 0. On each day, the mean per cent change is indicated by a plus sign (+).

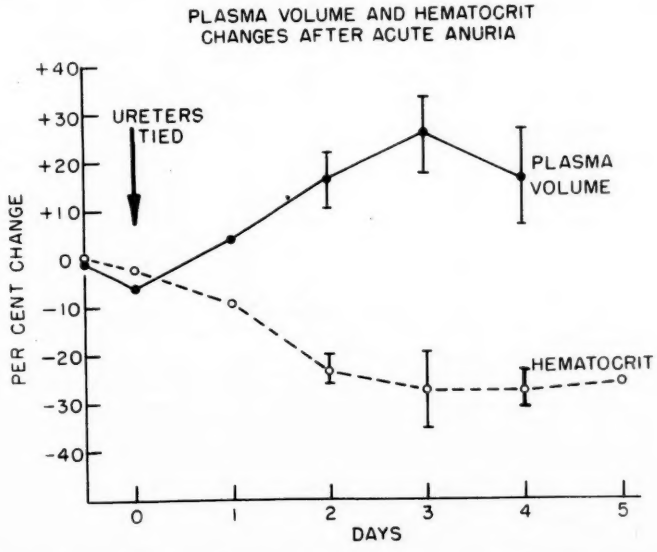



Fig. 2.—The mean per cent change in plasma volume as compared with the mean per cent change in hematocrit (volume of packed red cells) on each day following ureteral ligation. The range of one standard deviation of the means is indicated.

TABLE I. SUMMARY OF DATA

| EXPERIMENT | DAY | | PLASMA VOLUME ML. | PLASMA VOLUME % CHANGE | VOLUME PACKED R. B. C. % | VOLUME PACKED R. B. C. % CHANGE | WEIGHT LB. | MEQ./L. PLASMA NA | MEQ./L. PLASMA K | MEQ./L. PLASMA CL | CREATININE P | CREATININE MG./100 ML. PLASMA | BUN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | Preop. | | 737 | | 45 | | 37 | 145 | 4.3 | 108 | | | |
| | Postop. | 0 | | | 42 | -7 | | | | | | | |
| | | 1 | 1030 | +40 | 38 | -16 | | 146 | 4.4 | 118 | | | |
| | | 2 | 723 | -2 | 30 | -33 | | 133 | 4.7 | 103 | | | |
| | | 3 | 800 | +9 | 25 | -44 | | 135 | 7.8 | 100 | 15.6 | | |
| | | 4 | 763 | +3 | 23 | -48 | | 135 | 8.2 | 83 | | | |
| II | Preop. | | 700 | | 51 | | 54 | | | | | | |
| | Postop. | 0 | | | 53 | +3 | | | | | | | |
| | | 1 | 693 | -1 | 53 | +4 | | 149 | 4.5 | 122 | | | |
| | | 2 | 820 | +18 | 42 | -18 | | 148 | 7.1 | 113 | 9.7 | | |
| | | 3 | — | | | | | 149 | 7.9 | 105 | 15.9 | | |
| III | Preop. | | 598 | | 43 | | 29 | 148 | 4.3 | 116 | | | |
| | Postop. | 0 | 512 | -14 | 44 | +2 | | 148 | 4.3 | 117 | | | |
| | | 1 | 860 | +43 | 38 | -12 | 30 | 142 | 5.8 | 101 | | | |
| | | 2 | 1250 | +109 | 30 | -30 | 29 | 137 | 9.9 | 89 | 10.8 | | |
| | | 3 | 905 | +83 | 29 | -33 | 30 | 141 | 7.5 | 104 | 10.4 | | |
| IV | Preop. | | 575 | | 46 | | 29 | 153 | 5.3 | 110 | | | |
| | Postop. | 0 | 535 | -7 | 49 | +9 | | 146 | 6.1 | | | | |
| | | 1 | 604 | +5 | 42 | -8 | 29 | 145 | 5.9 | 120 | | | |
| | | 2 | 744 | +29 | 33 | -28 | | 137 | 7.5 | | 11.6 | | |
| V | Preop. | | 620 | | 51 | | | 152 | 4.0 | 117 | | | |
| | Postop. | 0 | 610 | -2 | 50 | -2 | 29 | 146 | 4.4 | 136 | | | |
| | | 1 | 650 | +5 | 40 | -22 | 29 | 140 | 4.3 | 101 | | | |
| | | 2 | 629 | +1 | 38 | -26 | 29 | 141 | 6.0 | 106 | | | |
| | | 3 | 655 | +6 | 38 | -26 | 29 | 131 | 8.3 | 95 | | | |
| VI | Preop. | | 365 | | 45 | | 18 | 142 | 4.9 | 108 | | | |
| | Postop. | 0 | 306 | -17 | 42 | -8 | | 140 | 4.3 | 109 | | | |
| | | 1 | 354 | -3 | 33 | -27 | 18 | 135 | 6.2 | 100 | 11.2 | | |
| | | 2 | 442 | +22 | 22 | -51 | 18 | 129 | 8.7 | 89 | 18.7 | | |
| *Above animals given food and water ad libitum* | | | | | | | | | | | | | |
| VII | Preop. | | — | | 49 | | 29 | 144 | 3.4 | 113 | 2.9 | | |
| | Postop. | 0 | 610 | | 47 | -5 | | 139 | 3.4 | 116 | 3.5 | | |
| | | 1 | 611 | 0 | 39 | -20 | 28 | 148 | 4.6 | 125 | 4.3 | | |
| | | 2 | 636 | +20 | 32 | -35 | 27 | 148 | 9.3 | 130 | 13.8 | | |
| VIII | Preop. | | 985 | | 45 | | 51 | 172 | 3.7 | 109 | 3.5 | | |
| | Postop. | 0 | 978 | -1 | 40 | -10 | | | 3.8 | 122 | 4.2 | | |
| | | 1 | 1038 | +5 | 35 | -20 | 50 | | 5.0 | 122 | 8.8 | | |
| | | 2 | 1274 | +29 | 33 | -25 | 48 | | 8.2 | 119 | 9.4 | | |
| | | 3 | 1090 | +11 | 32 | -27 | 47 | | 10.1 | 120 | 11.7 | | |
| IX | Preop. | | 918 | | 41 | | | 151 | 4.5 | 119 | 4.2 | | |
| | Postop. | 0 | 898 | -2 | 40 | -2 | 32 | 153 | 4.5 | 114 | 4.4 | | |
| | | 1 | 890 | -3 | 35 | -13 | 31 | 167 | 7.6 | 113 | 7.9 | | |
| | | 2 | 890 | -3 | 35 | -29 | 31 | 167 | 9.5 | 111 | 12.3 | | |
| X | Preop. | | 388 | | 43 | | 22 | 145 | 4.0 | 124 | 4.9 | | |
| | Postop. | 0 | 388 | 0 | 42 | -2 | | 145 | 4.1 | 113 | 5.0 | | |
| | | 1 | 417 | +8 | 34 | -21 | 19 | 140 | 6.8 | 113 | 9.6 | | |
| | | 2 | — | — | 27 | -37 | 17 | 143 | 8.2 | 115 | 16.5 | | |
| | | 3 | 465 | +20 | 25 | -42 | 17 | 140 | 9.4 | 101 | 24.0 | | |
| XI | Preop. | | 1116 | | 49 | | 64 | | | | | | |
| | Postop. | 1 | 1047 | -6 | 51 | +4 | 63 | | | | | | |
| | | 2 | 1127 | +1 | 45 | -8 | 63 | | | | | | |
| | | 3 | 1321 | +18 | 44 | -10 | 62 | | | | | | |
| | | 4 | 1123 | +1 | 42 | -14 | 60 | | | | | | |
| | | 5 | 1081 | -3 | 41 | -16 | 59 | | | | | | |

TABLE I—CONT'D

| EXPERIMENT | DAY | | PLASMA VOLUME ML. | PLASMA VOLUME % CHANGE | VOLUME PACKED R. B. C. % | VOLUME PACKED R. B. C. % CHANGE | WEIGHT LB. | MEQ./L. PLASMA NA | MEQ./L. PLASMA K | MEQ./L. PLASMA CL | P | CREATININE MG./100 ML. PLASMA | BUN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| XII | Preop. | | 591 | | 44 | | 25 | | | | | | |
| | Postop. | 1 | 602 | +2 | 43 | -2 | 23 | | | | | | |
| | | 2 | 610 | +3 | 39 | -11 | 22 | | | | | | |
| XIII | Preop. | | 892 | | 48 | | 43 | 164 | 4.5 | 116 | 3.6 | 1.2 | 11.5 |
| | Postop. | 1 | 760 | -15 | 49 | +1 | 42 | 156 | 4.2 | 116 | 5.5 | 5.7 | 27 |
| | | 2 | 923 | +3 | 46 | -4 | 42 | 156 | 4.5 | 114 | 7.3 | 8.3 | 61 |
| | | 3 | 1274 | +43 | 42 | -13 | 41 | 158 | 4.7 | 111 | 11.2 | 11.0 | 87 |
| | | 4 | 1440 | +61 | 40 | -17 | 39 | 162 | 5.4 | 111 | 15.1 | 13.8 | 123 |
| | | | | | | | | 164 | 7.3 | 111 | 18.7 | 15.3 | 184 |
| | | | | | | | | 165 | 8.5 | 106 | 23.6 | 18.5 | 215 |
| XIV | Preop. | | 447 | | 46 | | 22 | 159 | 4.4 | 126 | 6.1 | 1.0 | 2.5 |
| | Postop. | 1 | 441 | -1 | 45 | -2 | 21 | 152 | 4.6 | 115 | 8.6 | 4.4 | 51 |
| | | 2 | 601 | +34 | 34 | -26 | 20 | 146 | 6.1 | 116 | 10.9 | 7.6 | 102 |
| | | 3 | 702 | +57 | 30 | -35 | 20 | 150 | 7.4 | 111 | 14.8 | 11.6 | 180 |
| | | 4 | 665 | +49 | 28 | -39 | 19 | 147 | 9.9 | 105 | 17.4 | 13.2 | 203 |
| | | 5 | | | | | 18 | | | | | | |
| XV | Preop. | | 543 | | 44 | | 28 | 181 | 5.6 | 122 | 5.2 | 1.7 | 13.8 |
| | Postop. | 1 | 629 | +16 | 45 | +2 | 27 | 177 | 5.9 | 122 | 9.1 | 5.2 | 54.5 |
| | | 2 | 658 | +21 | 40 | -10 | 26 | 166 | 9.2 | 115 | 10.8 | 9.2 | 102 |
| | | 3 | 843 | +55 | 34 | -23 | 25 | 168 | 8.0 | 112 | 14.3 | 12.2 | 203 |
| | | 4 | 676 | +24 | 35 | -20 | 25 | 171 | 8.6 | 112 | 21.8 | 14.5 | 246 |
| XVI | Preop. | | 690 | | 51 | | 39 | | | 118 | 5.2 | 1.5 | |
| | Postop. | 1 | 658 | -5 | 48 | -6 | 37 | 159 | 4.3 | 118 | 6.5 | 5.9 | 39.5 |
| | | 2 | 529 | -23 | 45 | -12 | 37 | 159 | 5.4 | 113 | 9.0 | 10.2 | 110 |
| | | 3 | 560 | -19 | 41 | -20 | 36 | 157 | 5.7 | 112 | 10.5 | 12.6 | 172 |
| | | 4 | 550 | -20 | 38 | -26 | 35 | 162 | 6.7 | 109 | 14.8 | 16.1 | 212 |
| | | 5 | 464 | -33 | 39 | -24 | 35 | 160 | 7.4 | 108 | 19.6 | 18.3 | 315 |
| | | 6 | 506 | -27 | 41 | -20 | 33 | 156 | 9.2 | 100 | 22.0 | 20.0 | 365 |
| XVII | Preop. | | 364 | | 43 | | 20 | 153 | 3.5 | 110 | 5.5 | 1.1 | 15 |
| | Postop. | 1 | 310 | -15 | 42 | -2 | 20 | 146 | 3.8 | 110 | 11.2 | 4.8 | 52 |
| | | 2 | 360 | -1 | 36 | -16 | 19 | 154 | 5.0 | 108 | 15.3 | 7.8 | 108 |
| | | 3 | 367 | +1 | 31 | -28 | 18 | 153 | 5.9 | 104 | 22.0 | 11.3 | 186 |
| | | 4 | 332 | -9 | 30 | -30 | 18 | 153 | 7.5 | 101 | 24.5 | 13.5 | 228 |
| | | 5 | 409 | +12 | 29 | -33 | 17 | 153 | 10.1 | 94 | 27.6 | 15.7 | 392 |
| XVIII | Preop. | | 360 | | 55 | | 29 | 159 | 3.4 | 115 | 4.6 | 0.5 | 12.8 |
| | Postop. | 1 | 347 | -4 | 52 | -5 | 28 | 158 | 3.4 | 114 | 8.1 | 5.2 | 69 |
| | | 2 | 413 | +15 | 48 | -13 | 27 | 152 | 4.5 | 114 | 12.4 | 9.1 | 120 |
| | | 3 | 431 | +20 | 43 | -22 | 27 | 156 | 5.1 | 110 | 19.6 | 13.0 | 180 |
| | | 4 | 446 | +24 | 42 | -24 | 26 | 156 | 5.5 | 109 | 20.3 | 16.0 | 220 |
| | | 5 | 481 | +34 | 38 | -31 | 25 | 157 | 7.5 | 108 | 21.9 | 17.0 | 315 |
| | | 6 | 557 | +55 | 38 | -31 | 25 | 157 | 8.8 | 103 | 36.1 | 22.6 | 360 |

Animals I to VI were allowed food and water. Animals XI to XVIII had plasma volumes determined with $I^{131}$ human serum albumin, while Evans blue was used for plasma volume determinations in Animals I to X.

surviving animals was 25.3 per cent above the preoperative value. By the "t" test for the significance of the difference between the mean plasma volume on Day 0 and on the third postoperative day, P was found to be $< .02$.

Actually, the rise in plasma volume was somewhat greater, for the animals lost considerable blood in sampling. If this volume of plasma were taken into account, the mean rise in the plasma volume would be 30 to 35 per cent.

B. *Changes in the Volume of Packed Red Cells (VPRE).*—The drop in VPRC in all of the animals was quite consistent. On the third day the mean drop was about 27 per cent and stayed at this level until the animals suc-

cumbed (Fig. 2). This decline was present in every animal. The change in hematocrit from the day of surgery to the fourth postoperative day was highly significant statistically ($P < .01$).

C. *Electrolyte Changes.*—As can be seen from Table I, there was a pronounced rise in the plasma levels of potassium and inorganic phosphorus. In all of the animals studied, plasma levels of potassium prior to death were in the toxic range.

There was a fall in the plasma chloride level, averaging 14 meq. per liter in the group allowed access to food and water and 9 meq. per liter in the starved group. This difference between the two groups is not statistically significant, however, because of the small numbers of animals studied.

The changes in the plasma sodium level were not constant, although the "fed" group exhibited a greater tendency toward a drop, averaging 11 meq. per liter.

D. *Changes in Blood Urea Nitrogen and Creatinine Levels.*—As expected, the levels of these substances rose progressively until the animals expired.

E. *Pathologic Findings.*—The gross and microscopic changes observed at the autopsies of five dogs, which died three, two, four, three, and four days, respectively, following successful bilateral ureteral ligations, were similar with a few minor variations.

In every animal, the tissues appeared normally moist, but serosal and visceral surfaces showed conspicuous congestion. The most striking gross observation was engorgement and dilatation of the superior and inferior cavae and dilation of the right atrium and ventricle. The lungs showed congestion, edema, and alveolar hemorrhage. The livers exhibited acute central congestion and sinusoidal edema. The kidneys were swollen and the renal pelves and ureters proximal to the ligatures were dilated.

Microscopic changes beyond those of congestion, edema, and occasional capillary hemorrhages were not impressive. Some of the small vessels of the heart, stomach, and intestine showed slight swelling of medial muscle fibers. One dog had chronic pyelonephritis; another had focal acute inflammatory changes in the kidneys. Rare casts were seen. Except for general depletion of adrenal cortical lipid vascuoles, morphologic changes in endocrine organs, including parathyroids, were not observed.

## DISCUSSION

It is now well recognized that the immediate cause of death in acute renal failure is usually hyperkalemia or pulmonary edema from congestive heart failure. It is generally believed that the elevated serum potassium is a result of the liberation of this intracellular cation into the extracellular fluid with no avenue for its excretion. The elevated plasma volume and congestive heart failure have commonly been ascribed to overenthusiastic treatment with sodium-containing fluids. However, the data derived from the present study demonstrate that the plasma volume increases markedly in acute renal failure even when no exogenous fluid is supplied.

The increased plasma volume almost certainly results from transfer of intracellular water. Hamburger and Richet[13] and Swan and Merrill[14] have shown that acute anuria in man may be accompanied by an increase in total body water. The magnitude of the increment in body water was measured by the extra amount of urine passed by patients after cessation of anuria. The excess fluid liberated was observed to be about 10 per cent of the body weight. Metabolic water produced in particularly large quantities by catabolism of fat contributed largely to this increase. It should be noted (Table I) that Animals III, IV, V, VI, and IX showed no appreciable weight losses and many of the others had only insignificant decline in weight, especially since neither fluid nor tissue depletions were being replaced. The animals allowed fluid maintained their weight despite some vomiting, whereas the starved animals lost about 10 per cent of their body weight.

These observations have some bearing on the anemia of acute anuria. Muirhead and associates[15, 16] have presented evidence for the hemolytic nature of the anemia of acute renal insufficiency in rabbits and dogs. However, our data indicate that hemodilution must play a role. Although the per cent rise in plasma volume was about the same as the per cent fall in the volume of packed red cells (Fig. 2), this relationship was fortuitous. For example, a rise in plasma volume of 25 per cent with a volume of packed red cells of 50 per cent would cause the hematocrit to drop from 50/100 to 50/112.5, or 11 per cent.

Although these findings are derived from animal data, they indicate the necessity of rigorous fluid restriction in the management of patients with acute renal failure.

## SUMMARY

Acute anuria in dogs resulted in a rise in plasma volume averaging more than 25 per cent of normal on the third day after ureteral ligation even when the animals were given no food or fluids. There was a drop in volume of packed red cells of similar magnitude.

This increased plasma volume is partly responsible, by means of hemodilution for the anemia of acute renal failure.

We wish to thank Dr. Wallace N. Jensen and Miss Helen Ashenbrucker for performing the counting in the $I^{131}$ human serum-albumin plasma volume determinations; Dr. H. P. Hargreaves for assistance with the ureteral ligations; Mr. Gilbert S. Tanner, Mr. E. L. Simons, and Mr. Paul N. Sine for the chemical determinations; Dr. B. V. Jager for constructive criticism of the manuscript.

## REFERENCES

1. Stock, R. T.: Acute Urinary Suppression. Observations in Twenty-two Patients, Am. J. Med. **7**: 45-55, 1949.
2. Friedberg, Charles K.: Congestive Heart Failure of Renal Origin. Pathogenesis and Treatment of Four Cases of Carbon Tetrachloride Nephrosis, Am. J. Med. **9**: 164-174, 1950.
3. Iseri, L. T., Batchelor, T. M., Boyle, A. J., and Myers, G. B.: Studies of Fluid, Electrolyte and Nitrogen Balance in Acute Renal Insufficiency, A. M. A. Arch. Int. Med. **89**: 188-215, 1952.

4. Strauss, M. B.: Acute Renal Insufficiency Due to Lower-Nephron Nephrosis, New England J. Med. **239**: 693-700, 1948.
5. Editorial, Acute Anuria, Lancet **2**: 381-382, 1953.
6. Sirota, J. H., and Kroop, I.: Water and Electrolyte Disturbances in Acute Renal Insufficiency, Fed. Proc. **10**: 126, 1951.
7. Price, P. B., and Longmire, W. P.: The Use of T-1824 in Plasma Volume Determinations, Bull. John Hopkins Hosp. **71**: 51-83, 1942.
8. Schales, O., and Schales, S. S.: Simple and Accurate Method for Determination of Chloride in Biological Fluids, J. Biol. Chem. **140**: 879-884, 1941.
9. Fiske, C. H., and Subbarow, Y.: Colorimetric Determination of Phosphorus, J. Biol. Chem. **66**: 375-400, 1925.
10. Ormsby, A. A.: Direct Colorimetric Method for Determination of Urea in Blood and Urine, J. Biol. Chem. **146**: 595-604, 1942.
11. Phillips, R. A.: Creatinine Method, in Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry (Methods), Army ed., Baltimore, 1943, Williams & Wilkins Company, Vol. 2.
12. Fields, T., Kaplan, E., and Terril, M.: A Simplified Technique for Blood Volume Determinations Using $I^{131}$ HSA, J. Lab. & Clin. Med. **43**: 332-334, 1954.
13. Hamburger, J., and Richet, G.: Sur un phenomene de liberation d'eau endogene observé notamment au cours de certaines anuries, Bull. Soc. méd. hôp. Paris **68**: 368, 1952.
14. Swan, R. C., and Merrill, J. P.: The Clinical Course of Acute Renal Failure, Medicine **32**: 215-292, 1953.
15. Muirhead, E. E., Jones, F., Stirman, J. A., and Lesch, W.: Hemolytic Anemia of the Dog Following Bilateral Nephrectomy, Am. J. Physiol. **173**: 371-378, 1953.
16. Muirhead, E. E., Jones, F., and Grollman, A.: The Anemia of Renal Insufficiency as Induced by Bilateral Nephrectomy of the Rabbit: With Emphasis on Its Hemolytic Nature, J. Lab. & Clin. Med. **39**: 505-517, 1952.

# BODY COMPOSITION AND OXYGEN CONSUMPTION OF OVERWEIGHT, NORMAL, AND UNDERWEIGHT WOMEN

LOUIS C. JOHNSTON, M.D., AND LIONEL M. BERNSTEIN, M.D.
CHICAGO, ILL.

THIS investigation was undertaken to define the body composition of women and to describe its relationship to their oxygen consumption. The body was considered as composed of fat mass, extracellular fluid, cell mass, and bone mineral weight.[1, 2] Such data on composition of women of varying weight are scanty[1] although these figures are available for men.[1, 2] However, that women might be considerably different is suggested by recent evidence comparing men and women of standard weight and similar ages which describes women as having approximately twice the fat mass of men.[3] After considering this marked difference of fat mass and the well-known lowered oxygen requirement of women,[4] it was considered of interest to investigate the relationship of the percentage of fat of normal, overweight, and underweight individuals and the rate at which their nonfatty tissue, i.e., their "cell mass," consumed oxygen. The various previously mentioned elements of body composition in this investigation were all indirectly computed from determinations of antipyrine and inulin spaces, that is, from total body water and extracellular water, respectively, performed on seventeen subjects of varying age and weight.

## METHODS

A. *Subjects.*—All subjects were healthy women of constant body weight and between 21 and 59 years of age. All were occupied as either nurses or housewives and carried on their routine activities until they entered the hospital one day before the determinations. None had evidence of abnormal fluid depots.

B. *Direct Procedures.*—Total body water (TBW) was determined by antipyrine using the Beckman spectrophotometer.[5] Plasma samples were not taken before three hours and were corrected for plasma protein.[6] Extracellular water was measured by inulin.[7, 8] A five-hour constant infusion utilizing a Murphy drip[10] was found satisfactory. Oxygen consumption was measured in the basal state on the morning of the fluid determinations at the bedside by a clinical machine.* In all cases where this suggested abnormal thyroid function, radioactive iodine uptake studies were made, all of which were within the normal limits.[11]

C. *Calculations.*—Lean body mass (LBM) or the difference between total body weight and fat mass[12] was calculated from total body water by the formula of Pace and Rathbun[13] which assumes the LBM to be 73.2 per cent water; thus, in kilograms, LBM equals liters of total body water divided by 0.732. Cell mass (CM) or LBM minus the sum of the extracellular fluid and the minerals of the bone not included in the cell mass[1] was calculated from intracellular water, assuming the cells to be 70 per cent water.[2] Intracellular water is total body water minus extracellular water; thus, in kilograms CM equals liters of total body water minus liters of extracellular water divided by 0.70. Fat mass (FM)

From the Department of Medicine, University of Illinois, Chicago, Ill.
Submitted for presentation at the Twenty-seventh Annual Meeting of the Central Society for Clinical Research, 1954.
Received for publication Aug. 8, 1954.
*"Metabulator," Sanborn Company, Cambridge, Mass.

TABLE I. BASAL OXYGEN CONSUMPTION AND BODY COMPOSITION OF WOMEN

| SUBJECT | HEIGHT (CM.) | AGE (YR.) | TOTAL BODY WEIGHT (KG.) | PER CENT OF STANDARD BODY WEIGHT | EXTRACELLULAR WATER (L.) | TOTAL BODY WATER (L.) | CALCULATED FAT MASS | | CALCULATED LEAN BODY MASS (KG.) | CALCULATED CELL MASS (KG.) | SURFACE AREA (SQ. M.) | OXYGEN CONSUMED (ML./MIN.) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | METHOD A (KG.) | METHOD B (KG.) | | | | |
| 1 | 165 | 46 | 39.8 | 60.0 | 8.5 | 24.2 | 6.7 | 6.7 | 33.1 | 23.5 | 1.38 | 140 |
| 2 | 176 | 59 | 53.4 | 68.2 | 8.9 | 30.3 | 12.1 | 11.0 | 41.3 | 31.9 | 1.66 | 179 |
| 3 | 181 | 21 | 59.1 | 85.0 | 12.6 | 31.0 | 16.7 | 17.6 | 42.4 | 28.4 | 1.74 | 185 |
| 4 | 160 | 22 | 48.3 | 86.0 | 9.2 | 26.3 | 12.3 | 12.3 | 36.0 | 25.5 | 1.46 | 158 |
| 5 | 170 | 24 | 53.6 | 86.0 | 7.4 | 24.2 | 20.6 | 19.9 | 33.0 | 25.0 | 1.62 | 183 |
| 6 | 181 | 26 | 63.6 | 90.4 | 10.8 | 29.1 | 23.8 | 24.1 | 39.8 | 27.4 | 1.80 | 191 |
| 7 | 165 | 21 | 56.6 | 96.0 | 9.7 | 25.3 | 22.0 | 22.4 | 34.6 | 23.4 | 1.61 | 179 |
| 8 | 160 | 22 | 54.1 | 97.0 | 11.2 | 24.4 | 20.8 | 22.1 | 33.3 | 19.7 | 1.55 | 200 |
| 9 | 165 | 22 | 58.4 | 100.0 | 7.2 | 25.6 | 23.4 | 22.3 | 35.0 | 27.5 | 1.62 | 160 |
| 10 | 160 | 28 | 66.3 | 117.0 | 8.7 | 32.7 | 21.7 | 20.0 | 44.7 | 35.9 | 1.68 | 183 |
| 11 | 160 | 40 | 84.4 | 137.0 | 8.7 | 26.5 | 48.3 | 47.9 | 36.1 | 26.5 | 1.86 | 158 |
| 12 | 163 | 30 | 83.7 | 139.0 | 10.4 | 30.6 | 41.9 | 41.6 | 41.9 | 30.2 | 1.89 | 185 |
| 13 | 168 | 38 | 116.9 | 178.0 | 11.4 | 42.5 | 58.7 | 56.7 | 58.2 | 46.5 | 2.25 | 265 |
| 14 | 165 | 22 | 113.0 | 193.0 | 11.7 | 36.9 | 62.6 | 61.8 | 50.4 | 37.6 | 2.18 | 226 |
| 15 | 158 | 50 | 127.0 | 203.0 | 15.3 | 46.7 | 63.2 | 62.4 | 63.8 | 47.0 | 2.20 | 300 |
| 16 | 163 | 36 | 125.5 | 203.0 | 14.7 | 42.7 | 67.2 | 66.8 | 58.4 | 41.9 | 2.26 | 269 |
| 17 | 163 | 52 | 186.4 | 284.0 | 25.9 | 71.7 | 88.4 | 88.6 | 98.1 | 68.4 | 2.66 | 357 |

Composition, total body mass, kilograms

was determined in two ways. (A) FM is the difference between total body weight and lean body mass (FM equals TBW minus LBM). This is the method of Pace and Rathbun.[13] (B) FM is total body weight (TBW) minus extracellular fluid weight (ECFW) minus cell mass (CM) minus bone mineral weight (BMW). FM = TBW - ECFW - CM - BMW. ECFW is calculated as liters of extracellular (interstitial plus plasma) water multiplied by an assumed specific gravity of 1.012. CM is calculated as above. BMW is calculated as a constant (0.134) multiplied by the liters of intracellular fluid, the method of McCance and Widdowson[1] as modified by Keys and Brozek.[2] Surface area was computed by nomogram.[14] Standard weight was found from the 1953 standards of Metropolitan Life Insurance Company.

## RESULTS

The results are tabulated in Table I, where subjects are arranged from the lowest to highest percentage of standard body weight, and in Fig. 1 where the composition of each subject is graphically portrayed both in absolute terms and percentagewise. Total body weight ranged from 39.8 to 186.4 kilograms (87 to 410 pounds) or from 60 to 284 per cent of standard body weight for

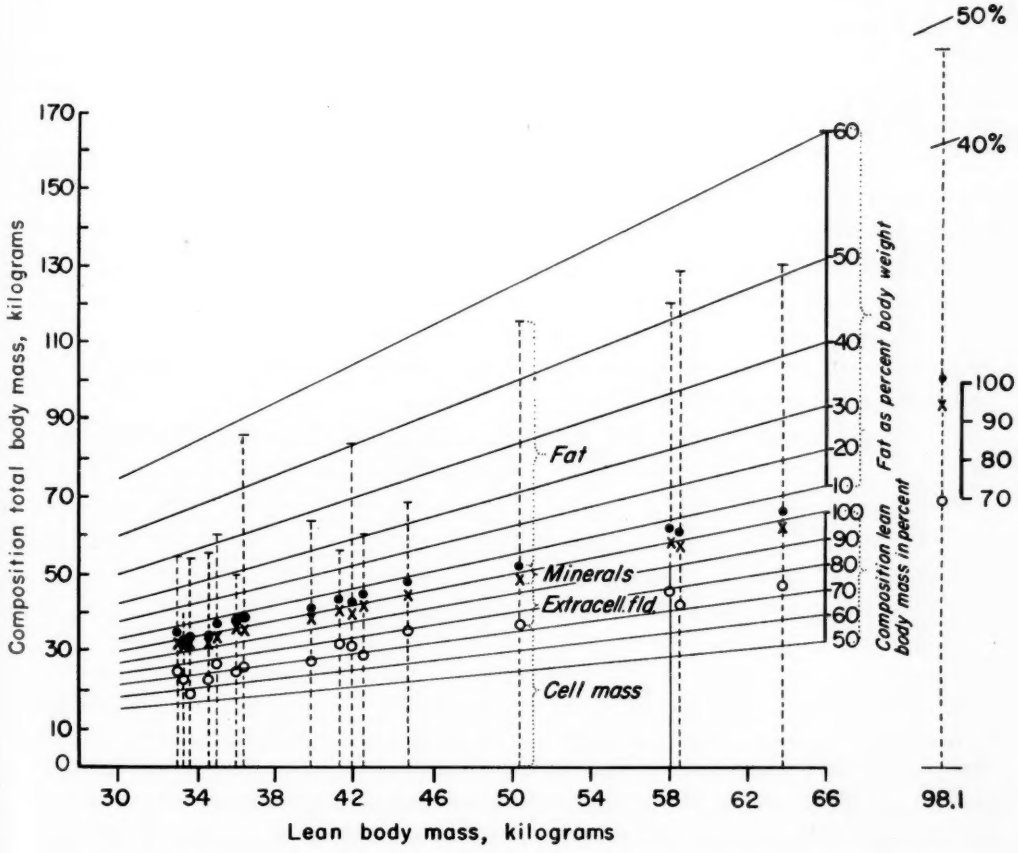



Fig. 1.—Body composition of women. Subjects arranged on abscissa as increasing lean body masses. Absolute composition is indicated as the ordinate on the left. On the right is the percentage composition of lean body mass. The "100% line" was drawn through points equal in magnitude to the antipyrine lean body mass determinations plotted on the abscissa and therefore does not quite equal the sum of the individually calculated components of the lean body mass—cell mass, extracellular fluid, and bone minerals. (The authors gratefully acknowledge the suggestions of Mr. Tohru Inouye in the preparation of this figure.)

height and age. The volume of extracellular water ranged from 7.2 to 25.9 L., or from 9.7 to 21.3 per cent body weight. The volume of extracellular water was more closely related to total body water (33.84 per cent) than to total body weight (14.88 per cent). The total body water varied from 24.2 to 71.7 L., or from 31.4 to 60.8 per cent body weight. Body fat as calculated by Method A varied from 6.7 to 88.4 kilograms, or from 16.9 to 57.2 per cent body weight. Body fat as calculated by Method B gave very similar values. The coefficient of correlation for calculation of body fat by these two methods was 0.99. The values used in this presentation are those of Method A.* Lean body mass ranged from 33.0 to 98.1 kilograms, or from 42.7 to 83.1 per cent body weight. Cell mass varied from 19.7 to 68.4 kilograms, or from 31.3 to 59.7 per cent body weight. Basal oxygen consumption ranged from 140 to 357 ml. per minute or from 85 to 136 ml. per square meter per minute.

## DISCUSSION

The composite presentation of data in Fig. 1 supports the opinion of others[13, 15, 16] that the constituents of the lean body mass remain of rather constant proportions throughout the weight range and that fat is the greatest independent variable. When allowance is made for the type of extracellular fluid indicator used, the results reported here for percentage composition of lean body mass (Fig. 1) lie within those ranges reviewed recently by Keeton.[16]

It has been suggested that when individuals of different age, different sex, or individuals in a state of changing weight are compared, the oxygen consumption is more closely correlated with lean body mass than with surface area, and still more closely with cell mass.[2] Others have noted that in young men of constant and approximately normal weight this closer correlation with lean body mass did not exist.[17, 18]

We have found that as the percentage of standard weight increased in a heterogeneous group of women, both lean body mass and the cell mass remained equally and highly correlated with the expanding surface area ($r$ equals 0.92 for both). (Fig. 2.) This caused the oxygen consumption of these women to remain equally well correlated with their surface area, their lean body mass, and their cell mass ($r$ equals 0.91, 0.94, and 0.92, respectively). (Fig. 3.)

*Fat Mass.*—The body fat of women of such markedly different weights was similar when calculated by the two different methods described (A and B). The coefficient of correlation between the two methods was 0.99. It would appear that in women body fat can be calculated equally well by either method. Our results are similar to those reported by others for normal women[2] and results reported by Steele and associates[19] for several obese women. The lower percentage of fat (13 to 28 per cent of body weight) in normal women mentioned in one report[1] is thought to be due to their utilization of different indicators of total and extracellular water that are in current use.

*The body fat was determined in one additional subject weighing 200 kilograms by the deuterium method. Her body fat was 62.5 per cent of body weight. We wish to thank Dr. Smith Freeman, Veterans Administration Hospital, Hines, Ill., for this determination.

In the past it was thought that the weight gain accompanying obesity could be completely accounted for by gain of metabolically inert lipid material and that cell mass did not increase.[20-22] This was founded upon the observation that the obese could be maintained in nitrogen balance on a reduction diet of 440 calories with a protein intake of one gram per kilogram of ideal weight, just like the nonobese,[20, 21] and that the creatinine coefficient of the obese person is normal when expressed as creatinine excreted per unit of ideal weight, whereas it is low when expressed in terms of actual weight.[21, 22] Further evidence for this viewpoint was recently contributed when it was shown in the experimental animal by the anoxia test that acute weight gain could be completely accounted for by gain of only fat and not cell mass.[23]

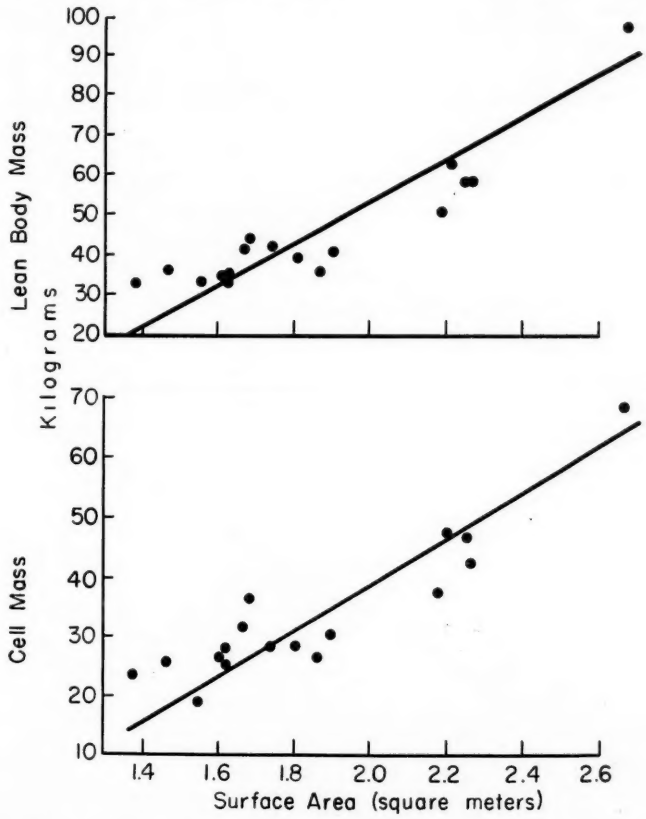



Fig. 2.—Relationship of lean body mass and cell mass to surface area. LBM = 52.6 SA – 51.3 (P = <.001); CM = 38.4 SA – 37.7 (P = <.001).

Behnke,[24] however, using specific gravity technique, showed that athletes may be grossly "overweight" without increases in per cent body fat. Keys and Brozek[2] suggested that weight gain in nonathletic individuals is composed of cell mass and extracellular fluid as well as fat. Their conclusion, based on data from males, was that the weight gained, or "obesity tissue," was composed of 24 per cent cell mass, 14 per cent extracellular fluid, and 62 per cent fat.

Our data show an unequivocal increase in cell mass accompanying obesity (Table I). An attempt to delineate the fat content of the "obesity tissue" of the women in this series was made. The application of this concept to women has not been published to our knowledge. Despite the wide range of age, and

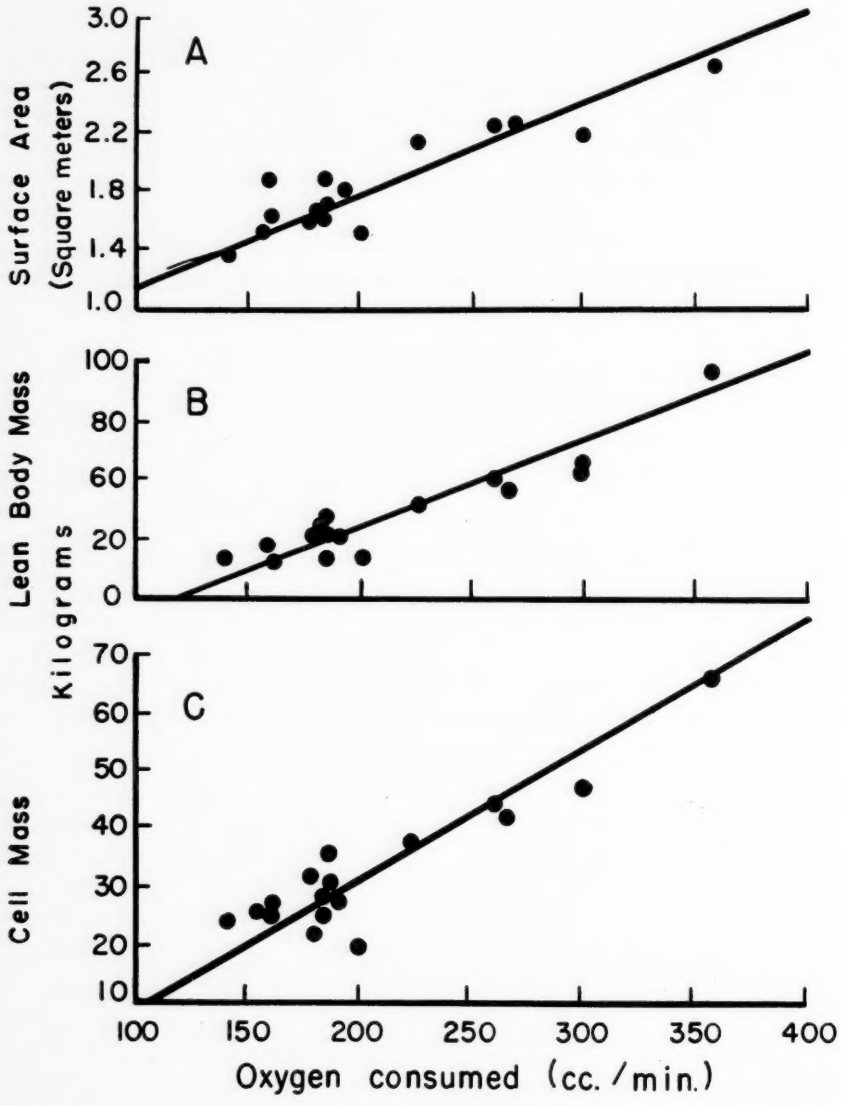



Fig. 3.—Relationship of surface area, lean body mass, and cell mass to basal oxygen consumption. SA = .0065 $O_2$ + 0.51 (P = <.001); LBM = 0.30 $O_2$ – 16.6 (P = <.001); CM = 0.23 $O_2$ – 13.6 (P = <.001).

despite the recognized fact that these are single studies on many individuals rather than serial studies on the same individual, it was believed that the data could partially answer the question of what is the fat content of "obesity tissue" in women. The per cent of standard weight was plotted against per

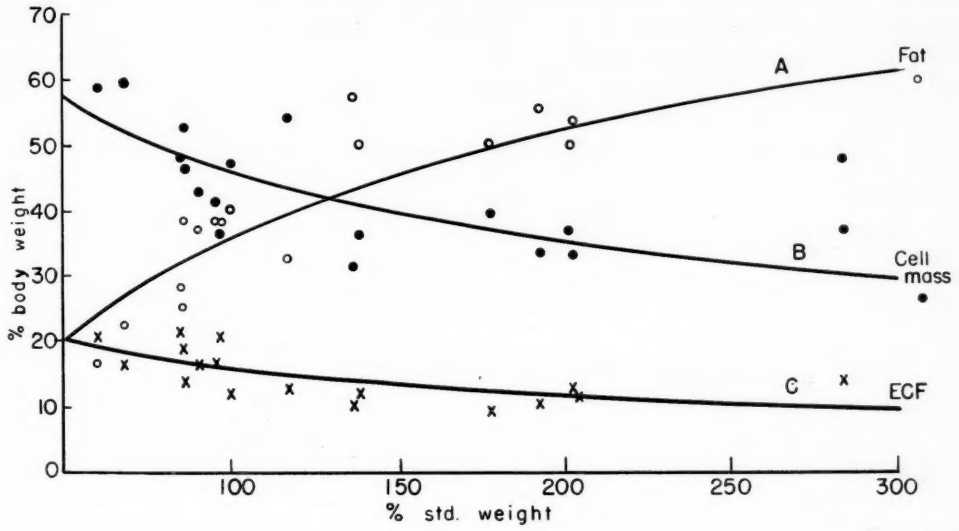



Fig. 4.—Relationships of fat, cell mass, and extracellular fluid to per cent standard weight (SW). Per cent Fat = – 70.0 plus 53.3 log. SW (P = <.001; Per cent CM = 118.2 – 36.1 log. SW (P = <.001); Per cent ECF = 45.7 – 14.8 log. SW (P = <.001).

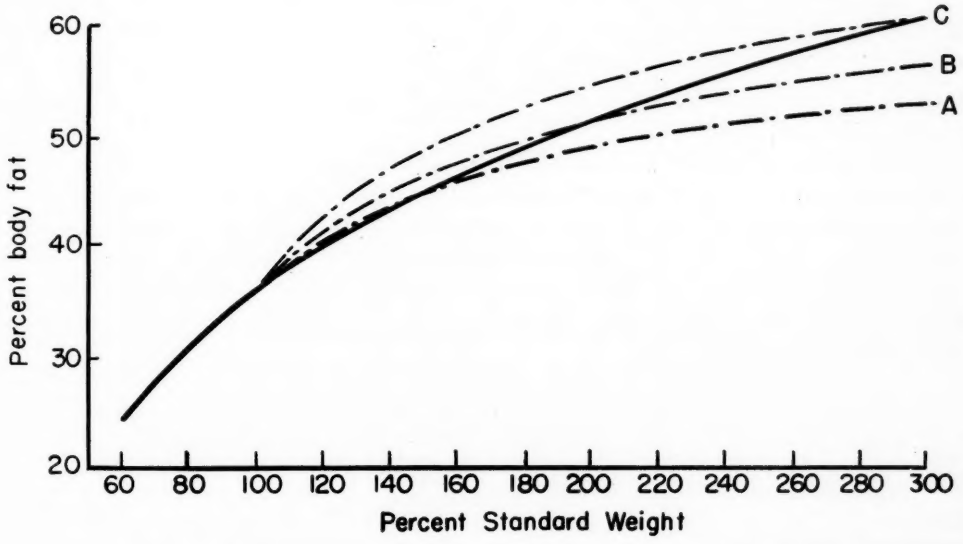



Fig. 5.—Observed relationship of per cent body fat to per cent standard body weight (solid line). The per cent body fat resulting from addition of "obesity tissue" to the normal 100 per cent standard weight individual is shown: Line *A* = 62 per cent fat; Line *B* = 67 per cent fat; Line *C* = 73.5 per cent fat.

cent body fat. A curvilinear relationship was recognized (Fig. 4, Line *A*) indicating that the increasing fat percentage tends to slacken in the more obese group. When this line was rectified by using the logarithm of the per cent standard weight and analyzed by the analysis of variance technique, the linear regression between logarithm of per cent standard weight and per cent body fat was very highly significant ($P < 0.001$).

TABLE II. RELATIONSHIP OF LOGARITHM OF PER CENT STANDARD WEIGHT AND PER CENT BODY FAT

| VARIANCE SOURCE | DEGREES FREEDOM | SUMS OF SQUARES | MEAN SQUARE | F |
|---|---|---|---|---|
| Regression | 1 | 2012.68 | 2012.68 | 46.72*** |
| Deviation from regression | 16 | 689.32 | 43.08 | |
| Total | 17 | 2702.00 | | |

From Fig. 4 the percentage of fat of normal women was taken as that percentage corresponding to 100 per cent standard weight, or 36 per cent fat (Fig. 5). To this normal reference body increasing amounts of "obesity tissue" with 62 per cent fat were added hypothetically. The resulting composition is shown as Line *A*, Fig. 5. The same was done for "obesity tissue" of 67 per cent fat (Line *B*) and 73.5 per cent fat (Line *C*). Though none of the hypothetical lines exactly coincide with the observed data, it is evident that "obesity tissue" has a fat content somewhere between 62 and 73.5 per cent fat. "Obesity tissue" with 67 per cent fat gives the predicted values which follow the observed total body fat percentage best in this series.

Thus, the data suggest that obesity tissue, in women, has a fat content similar to, but slightly greater than, that reported by Keys and Brozek[2] in men. Obviously, this is only an estimate which will be modified as larger series of individuals, studied in this fashion, become available and permit more accurate estimates.

*Cell Mass.*—The per cent of standard weight was found to be related to cell mass also in a curvilinear fashion (Fig. 4, Line *B*), thus indicating a decremental decrease of percentage cell mass accompanying increasing obesity. Rectification of the line was again accomplished by the logarithmic transformation of the per cent standard weight and the linear regression between logarithm of per cent standard weight and per cent cell mass was very highly significant ($P < .001$).

TABLE III. RELATIONSHIP OF LOGARITHM OF PER CENT STANDARD WEIGHT AND PER CENT CELL MASS

| VARIANCE SOURCE | DEGREES FREEDOM | SUMS OF SQUARES | MEAN SQUARE | F |
|---|---|---|---|---|
| Regression | 1 | 753.12 | 753.12 | 21.66*** |
| Deviation from regession | 16 | 556.20 | 34.76 | |
| Total | 17 | 1309.32 | | |

From Fig. 4, the percentage of cell mass of normal women was taken as that percentage corresponding to 100 per cent standard weight, or 46 per cent. "Obesity tissue" of different per cent cell mass was hypothetically added as

previously described to delineate the fat content of "obesity tissue." The value which best predicts the observed data was 24 per cent cell mass, the identical figure suggested by Keys and Brozek for men.[2]

*Extracellular Fluid.*—The relation of extracellular fluid to per cent standard weight was treated in a manner similar to that for body fat and cell mass (Fig. 4, Line *C*), and the linear regression between logarithm of per cent standard weight and per cent cell mass was very highly significant ($P < .001$).

TABLE IV. RELATIONSHIP OF LOGARITHM OF PER CENT STANDARD WEIGHT AND PER CENT EXTRACELLULAR FLUID

| VARIANCE SOURCE | DEGREES FREEDOM | SUMS OF SQUARES | MEAN SQUARE | F |
|---|---|---|---|---|
| Regression | 1 | 127.01 | 127.01 | 16.4*** |
| Deviation from regession | 16 | 123.66 | 7.73 | |
| Total | 17 | 250.67 | | |

With increasing standard weight, the extracellular fluid as per cent body weight decreases. A similar decrease in extracellular fluid as per cent of body weight noted with increasing standard weight is shown also in the data of McCance and Widdowson.[1] The observed decrease in extracellular fluid as per cent body weight could not occur with addition of "obesity tissue" with 14 per cent extracellular fluid as in males,[2] but would require "obesity tissue" containing less extracellular fluid. Hypothetical addition of "obesity tissue" of different per cents extracellular fluid to the 100 per cent standard weight individual with 16 per cent extracellular fluid, indicated that "obesity tissue" of 6 per cent extracellular fluid most accurately represented our data.

We found no significant relationship between the per cent standard weight and ratio of extracellular to total body water which suggests that, with increasing obesity, there is proportional decrease in both per cent extracellular fluid and per cent cell water.

## SUMMARY

The various components of body structure and their interrelations of seventeen women ranging from 60 per cent to 284 per cent of standard weight were studied. Surface area was found to be equally well correlated with both cell mass and lean body mass. Oxygen consumption was found to be equally well correlated with surfac area, lean body mass, and cell mass.

The composition of "obesity tissue" in women was calculated. This was found to contain 24 per cent cell mass, 6 per cent extracellular fluid, and 67+ per cent fat.

## REFERENCES

1. McCance, R. A., and Widdowson, E. M.: A Method of Breaking Down the Body Weights of Living Persons Into Terms of Extracellular Fluid, Cell Mass and Fat and Some Applications of It to Physiology and Medicine, Proc. Roy. Soc., London, s.B. **138**: 115-130, 1951.
2. Keys, A., and Brozek, J.: Body Fat in Adult Man, Physiol. Rev. **33**: 245-325, 1953.
3. Brozek, J., Chen, K. P., Carlson, W., and Bronczyk, F.: Age and Sex Differences in Man's Fat Content During Maturity, Fed. Proc. **12**: 21, 1953.
4. DuBois, E. F.: Basal Metabolism in Health and Disease, ed. 3, Philadelphia, 1936, Lea & Febiger.

5. Soberman, R., Brodie, B. B., Levy, B. B., Axelrod, J., Hollander, V., and Steele, J. M.: Use of Antipyrine in the Measurement of Total Body Water in Man, J. Biol. Chem. **179:** 31-42, 1949.
6. Deane, N., Schreiner, G. E., and Robertson, J. S.: Velocity of Distribution of Sucrose Between Plasma and Interstitial Fluid With Reference to Use of Sucrose for Measurement of Extracellular Fluid in Man, J. Clin. Invest. **30:** 1463-1468, 1951.
7. Gaudino, M., Schwartz, I. L., and Levitt, M. E.: Inulin Volume of Distribution as a Measure of Extracellular Fluid in Dog and Man, Proc. Soc. Exper. Biol. & Med. **68:** 507-511, 1948.
8. Gaudino, M., and Levitt, M. F.: Inulin Space as a Measure of Extracellular Fluid, Am. J. Physiol. **157:** 387-393, 1949.
9. Schwartz, I. L.: Measurement of Extracellular Fluid by Means of a Constant Infusion Technique Without Collection of Urine, Am. J. Physiol. **160:** 526-531, 1950.
10. Schwartz, I. L., Schacter, D., and Frienkel, N.: Measurement of Extracellular Fluid in Man by Means of a Constant Infusion Technique, J. Clin. Invest. **28:** 1117-1125, 1949.
11. Albright, E. C., and Gordon, E. S.: Diagnostic Use of Radioactive Iodine: Report of 1,000 Cases, J. Lab. & Clin. Med. **40:** 776, 1952.
12. Behnke, A. R.: Physiologic Studies Pertaining to Deep Sea Diving and Aviation, Especially in Relation to the Fat Content and Composition of the Human Body, Harvey Lect. **37:** 198-226, 1941-1942.
13. Pace, N., and Rathbun, E. N.: Studies on Body Composition: III. The Body Water and Chemically Combined Nitrogen Content in Relation to Fat Content, J. Biol. Chem. **158:** 685-691, 1945.
14. Boothby, W. M., Berkson, J., and Dunn, H. L.: Studies of the Energy Metabolism of Normal Individuals: A Standard for Basal Metabolism, With A Nomogram for Clinical Application, Am. J. Physiol. **116:** 468-484, 1936.
15. Edelman, I. S., Haley, H. B., Schloerb, P. R., Sheldon, D. B., Frieshanson, B. J., Stoll, G., and Moore, F. D.: Further Observations on Total Body Water: I. Normal Values Throughout the Life Span, Surg. Gynec. & Obst. **95:** 1-12, 1952.
16. Keeton, R. W.: Nutrition and Appetite Training During Illness, J. A. M. A. **151:** 253-260, 1953.
17. Miller, A. T., Jr., and Blyth, C. S.: Lean Body Mass as a Metabolic Reference Standard, J. Appl. Physiol. **5:** 311-316, 1953.
18. Best, W. R., Kuhl, W. J., and Consolazio, C. F.: Comparison of Physical and Metabolic Estimates of Active Protoplasmic Mass, Fed. Proc. **12:** 178, 1953.
19. Steele, J. M., Berger, E. Y., Dunning, M. F., and Brodie, B. B.: Total Body Water in Man, Am. J. Physiol. **162:** 313-317, 1950.
20. Strang, J. M., McClugage, H. B., and Evans, F. A.: The Nitrogen Balance During Dietary Correction of Obesity, Am. J. M. Sc. **181:** 336-349, 1931.
21. Duncan, G. G.: Diseases of Metabolism, ed. 3, Philadelphia, 1952, W. B. Saunders Company. (See chapter on Obesity by F. Evans, page 601).
22. McClugage, H. G., Booth, G., and Evans, F. A.: Creatinine Excretion in Abnormal States of Nutrition, Am. J. M. Sc. **181:** 349-355, 1939.
23. Goldberg, R. C., and Mayer, J.: Normal Iodine and Anoxia Resistance Accompanying Apparent Hypometabolism in Hereditary Obese Hyperglycemia Syndrome, Proc. Soc. Exper. Biol. & Med. **81:** 323-324, 1952.
24. Behnke, A. R., Feen, B. G., and Welham, W. C.: Specific Gravity of Healthy Men, J. A. M. A. **118:** 495-498, 1942.

# THE NONPROTEIN SOLUBLE SULFHYDRYL CONTENT OF HUMAN LEUKOCYTES AND ERYTHROCYTES IN INFECTION AND LEUKEMIA

ROSALIE GREEN AND SAMUEL P. MARTIN,* M.D.
DURHAM, N. C.

A RELATIONSHIP between cell proliferation and the concentration of nonprotein soluble sulfhydryl (NPSSH) in the cell environment was shown by Hammet[1] and Baker.[2] Subsequent work has shown the correlation between cell multiplication, cell age, and sulfhydryl content in bacteria, amoebae, eggs, sperm, and tumors.[3-9] Cantopoulos and Anderson studied various blood dyscrasias and noted a high NPSSH level in blood cells.[10]

The present study deals with a refined technique for the measurement of the nonprotein soluble sulfhydryl content in leukocytes and erythrocytes. Studies were conducted on normal individuals, individuals with acute infections, and individuals with various blood dyscrasias.

## METHOD

Approximately 60 ml. of blood was collected from fasting subjects using siliconized syringes and 0.02 mg. of heparin per milliliter as an anticoagulant.[11] The blood was processed in containers having a nonwetting surface and without exposure to excessive force due to acceleration. All glassware was rinsed with demineralized water. Dextran,† in a final concentration of 1 per cent, was added to induce rouleaux formation and rapid sedimentation of the red cells. The supernatant plasma containing predominantly white cells, and the sediment containing predominantly red cells were transferred to calibrated centrifuge tubes and the total volumes were noted. Counts were made of the red and white cell concentrations in each tube. The cells were concentrated by centrifugation at 1,000 x G for ten minutes and the packed volumes were recorded. After removing the supernatant fluids, the cells were deproteinized with 2.5 per cent sulfosalicylic acid (6.6 ml. per milliliter of packed cells), homogenized,‡ and filtered in the cold. One hundred milligrams of disodium versenate were added to the filtrate as a chelating agent. Amperometric titrations were done with 0.001 M silver nitrate using a modification of the method of Benesch and Benesch.[12]

In order to account for the red cell concentration in the white cell pack and the converse, the following simultaneous equation was used, solving for x and y.

$$a\,x + b\,y = A$$
$$c\,x + d\,y = B$$

Where the total count is equivalent to the cell count times the plasma volume in milliliters:

a = total count of leukocytes in white cell pack;
b = total count of erythrocytes in white cell pack;
c = total count of leukocytes in red cell pack;

---

From the Departments of Medicine and Bacteriology, Duke University School of Medicine, Durham, N. C.

This investigation was supported in part by a research grant G. 3476 of the National Institutes of Health, Public Health Service, by the Baxter Laboratories, The Spanel Foundation and the Duke Research Council.

Received for publication Aug. 9, 1954.

*Markle Scholar in Medicine.

†This dextran was pyrogen-free and was supplied by Pharmacia, Ltd., and the Commercial Solvents Corporation.

‡Tenbroeck ground glass tissue grinder.

d = total count of erythrocytes in red cell pack;
A = NPSSH measured in the W.B.C. pack;
B = NPSSH measured in the R.B.C. pack.

The unknown x and y were the white and red cells SH, respectively. The values were expressed as milligrams per $10^{10}$ cells.

## RESULTS

The sulfhydryl content of the leukocyte was greater than that of the erythrocyte (Table I). The NPSSH content of the white cell fluctuated over an eightfold range when compared with the control; that of the red cell remained relatively constant. Marked elevations in NPSSH content of the leukocyte occurred in chronic myelogenous leukemia, infectious disease, and myeloid metaplasia. Decreases were found in acute myeloblastic leukemia and in lymphocytic leukemia. The nonprotein soluble thiol content of the cell-free plasma in four subjects was too low to be measured by this method.

TABLE I. NONPROTEIN SOLUBLE SULFHYDRYL CONCENTRATION OF LEUKOCYTES AND ERYTHROCYTES IN UNTREATED SUBJECTS

| SUBJECT | NO. | W.B.C. MG. NPSSH | R.B.C. MG. NPSSH |
|---|---|---|---|
| Control | 6 | 1.63 ± 0.241 | 0.52 ± 0.041 |
| Chronic myelogenous leukemia | 7 | 3.3 ± 0.429 | 0.55 ± 0.029 |
| Myeloid metaplasia | 1 | 3.4 | 0.52 |
| Infectious disease | 6 | 4.2 ± 0.302 | 0.46 ± 0.038 |
| Lymphocytic leukemia | 5 | 1.8 ± 0.536 | 0.43 ± 0.089 |
| Myeloblastic leukemia | 2 | 0.9 | 0.31 |

## DISCUSSION

To obtain consistent results in normal subjects requires careful attention to two points. The cells must be handled rapidly with minimal trauma and limited exposure to room temperature. When blood cells were allowed to stand at room temperature before deproteinization, there were more marked fluctuations in the NPSSH level. Another factor is careful allowance for contamination of red and white cell samples with the opposite cell type. Marked variations were noted when these corrections had not been made.

Since there was no measurable NPSSH in the plasma, the level in whole blood reflects that of the cell content. Variations which occurred in cell numbers, in degree of differentiation, and in red and white cell ratios influenced the distribution of NPSSH in whole blood. It would seem advisable to report these values in reference to cell numbers and types.

The NPSSH of the normal leukocytes averaged 1.63 mg. ± 0.241 in $10^{10}$ cells and that of the erythrocyte averaged 0.52 mg. ± 0.041 in $10^{10}$ cells. Hardin and co-workers,[13] using the nitroprusside method, reported values of NPSSH of 5.1 and 0.72 mg. in $10^{10}$ W.B.C. and R.B.C., respectively, expressed as milligrams of glutathione in $10^{10}$ cells. Expressing their values as milligrams of GSH per 100 Gm. of cells, wet weight, Cantopoulos and Anderson[10] found the nor-

mal W.B.C. averaged 3.9 mg. per 100 Gm. and the normal R.B.C., 16.0 mg. per 100 Gm. The reasons for these discrepancies are not clear although different methods and techniques of cell separation were used.

In this series the normal leukocyte had two and one-half times greater NPSSH content than the erythrocyte. Leukocytes in infection, as well as in chronic myelocytic blood dyscrasias, were associated with increased NPSSH levels of the white blood cell with no change in that of the red blood cell. Cantopoulos and Anderson,[10] using the method of Binet and Weller, reported a high NPSSH in leukemic myelocytes. Hardin and co-workers,[13] using a colorimetric reaction, reported no change. In using an amperometric titration there is elevation of NPSSH in leukocytes of infection and metaplasia. These differences may be in the methods.

There was marked elevation of NPSSH in the groups with chronic myelogenous leukemia, myeloid metaplasia, as well as acute infectious diseases. In all three conditions, there were immature cells in the peripheral blood. There appears to be no difference in NPSSH content between the differentiated neoplastic cell and the cell of acute infection. In the blood of two patients containing blast cells of the myelogenous series and associated with acute leukemias, the NPSSH content was not elevated. These immature cells showed marked restriction in their differentiation. The early work of Hammet,[14] Baker[2] and Rapkine, as summarized in Barron,[15] as well as the later work of Barron,[15] indicates a relationship between NPSSH and cell growth. Cells not undergoing normal differentiation may have a lower sulfhydryl level.

In the group with lymphatic leukemia there was no significant elevation of NPSSH in white cells. Dietz and Steinberg[16] showed that the sulfate sulfur of rib marrow was not increased in lymphatic leukemia, but was increased in myelocytic leukemia. In their study of benzene-leukopenic rabbits, Parker and Kracke[17] found a marked decrease in the reduced glutathione of the blood and bone marrow.

## SUMMARY

Procedures for the rapid processing and separation of blood cells with minimal trauma have been described.

By amperometric titration with silver nitrate, the normal human erythrocyte contained 0.52 mg. NPSSH per $10^{10}$ cells ± 0.041. The normal human leukocyte contained 1.63 mg. NPSSH per $10^{10}$ cells ± 0.241.

There were marked rises in NPSSH content of the white blood cells, with no accompanying rise in that of the red blood cells in untreated individuals with chronic myelogenous leukemia, myeloid metaplasia, and acute infectious disease.

## REFERENCES

1. Hammet, F. S.: The Chemical Stimulus Essential for Growth by Increase in Cell Number, Protoplasm **7**: 297, 1929.
2. Baker, L. E.: The Chemical Nature of the Substances Required for Cell Multiplication, J. Exper. Med. **49**: 163, 1929.
3. Voegtlin, C., and Chalkley, H. W.: The Chemistry of Cell Division; the Effect of Glutathione on Cell Division in Amoeba Proteus, Pub. Health Rep. **45**: 3041, 1930.

4. Voegtlin, C.: Observations Concerning the Chemistry of Cell Growth and Division, Cold Spring Harbor Symp. Quant. Biol. **2:** 84, 1934.
5. Rane, L., and Subba Row, Y.: Studies on the Nutritional Requirements of Hemolytic Streptococci, Proc. Soc. Exper. Biol. & Med. **38:** 837, 1938.
6. Bodine, J. H., and West, W. L.: Changes in Metabolic Relations Between Yolk and Embryo During Embryonic Development, Fed. Proc. **13:** 14, 1954.
7. Barron, E. S. G., and Singer, T. P.: Enzyme Systems Containing Active Sulfhydryl Groups. The Role of Glutathione, Science **97:** 356, 1943.
8. Schacter, B., Entenman, C., and Shimkin, M. B.: Blood Plasma Sulfhydryl Levels During Growth and Regression of the Murphy Lymphosarcoma of the Rat, J. Nat. Cancer Inst. **13:** 647, 1952.
9. Barron, E. S. G., Nelson, L., and Ardao, M. I.: Regulatory Mechanisms of Cellular Respiration. II. The Role of Soluble Sulfhydryl Groups as Shown by the Effect of Sulfhydryl Reagents on the Respiration of Sea-Urchin Sperm, J. Gen. Physiol. **32:** 179, 1948.
10. Cantopoulos, A. N., and Anderson, H. H.: Sulfhydryl Content of Blood Cells in Dyscrasias, J. LAB. & CLIN. MED. **36:** 929, 1950.
11. McKinney, G. R., Martin, S. P., Rundles, R. W., and Green, R.: Respiratory and Glycolytic Activities of Human Leukocytes *in vitro*, J. Appl. Physiol. **5:** 335, 1953.
12. Benesch, R., and Benesch, R. E.: Amperometric Titration of Sulfhydryl Groups in Amino Acids and Proteins, Arch. Biochem. **19:** 35, 1948.
13. Hardin, B., Valentine, W. N., Follette, J. H., and Laurence, J. S.: Studies on the Sulfhydryl Content of Human Leukocytes and Erythrocytes, Am. J. Med. Sc. **228:** 73, 1954.
14. Hammet, F. S.: Possible Explanation of the Function of Glutathione in Developmental Growth, Science **79:** 457, 1934.
15. Barron, E. S. G.: Effect of Ionizing Radiations on Sulfhydryl Systems, Conf. Biol. Antioxidants **5:** 81, 1950.
16. Dietz, A. A., and Steinberg, A.: Chemical Composition of Normal and Leukemic Bone Marrow. The Role of Inorganic Sulfate in the Metabolism of Granulocytes, Atlantic City, N. J., 1949, Div. of Biol. Chem., American Cancer Society, Inc., p. 70.
17. Parker, F. P., and Kracke, R. P.: Further Studies on Experimental Granulopenia, Am. J. Clin. Path. **6:** 41, 1936.

# PROPERTIES OF PLASMA THROMBOPLASTIN ANTECEDENT (PTA) IN RELATION TO BLOOD COAGULATION

ROBERT L. ROSENTHAL, M.D.
NEW YORK, N. Y.

WITH THE TECHNICAL ASSISTANCE OF ESTHER GENDELMAN, B.S.

PLASMA thromboplastin antecedent (PTA) deficiency was discovered in two sisters and their maternal uncle who had a moderately severe hemophilia-like disease.[1] These subjects were found to have normal amounts of the other known plasma thromboplastin factors; antihemophilic globulin (AHG) and plasma thromboplastin component (PTC),[2] in their blood. This paper presents observations on various properties of PTA.

## METHODS

The measurement of PTA activity of a sample is made by measuring the sample's ability to correct the clotting defect of a PTA-deficient subject. In the analysis, 1 ml. of freshly drawn PTA-deficient blood is added to 0.05 or 0.1 ml. of the test material and the corrective effect upon the clotting time and prothrombin utilization is measured by methods previously described.[3] The results are interpreted in relation to control values obtained by either no addition to the PTA-deficient blood or by the addition of 0.1 ml. of 0.85 per cent NaCl solution. Both techniques give similar results. In a few of the earlier studies PTA activity was measured by the ability of a test substance to reduce the recalcified plasma clotting time of PTA-deficient plasma. Similar studies were performed on AHG- and PTC-deficient samples as indicated in the results. Plasma and serum have been adsorbed by $BaSO_4$ by the method described by Rosenfield and Tuft.[4] The $BaSO_4$ wash was obtained by resuspending the $BaSO_4$, following adsorption of either plasma or serum, in 0.85 per cent NaCl, one-third the original plasma volume. After removal of the NaCl wash by centrifugation, the $BaSO_4$ was suspended in 3 per cent sodium citrate, one-third the original plasma volume, in order to obtain the citrate eluate.[5, 6] The eluate was then dialyzed overnight against 0.85 per cent NaCl. Cohn fractions* were reconstituted in 0.85 per cent NaCl and 3 per cent sodium citrate as described by White, Aggeler, and Emery.[7] Fractions of normal serum separated by paper electrophoresis[8, 9] were obtained as follows: about 0.2 ml. of normal serum was placed on a filter paper strip 2½ inches wide. Runs were made for 5 hours, using a barbiturate buffer of pH 8.6 and 0.05 molar strength at 200 volts. At the end of the run, a small guide strip was cut off the record and stained with bromphenol blue. With the use of the guide strip, the filter paper was cut into several segments, each of which was placed in 0.5 ml. of 0.85 per cent NaCl in order to elute the protein fractions. Seitz-filtered plasma was prepared by a single passage of 5 to 10 ml. quantities of plasma through either 20 or 50 per cent asbestos filters. Precipitates obtained by various degrees of saturation of normal plasma with ammonium sulfate were redissolved in 0.85 per cent NaCl, one-half the original plasma volume, and dialyzed against distilled water until the sulfate ion was completely removed.

From the Laboratories of the Joseph and Helen Yeamans Levy Foundation, Beth Israel Hospital, New York, N. Y.

Supported in part by a grant (H-1107) from the National Heart Institute, National Institutes of Health, U. S. Public Health Service.

Received for publication Aug. 12, 1954.

*Cohn fractions were provided through the courtesy of Dr. F. F. Johnson of Cutter Laboratories, Berkeley, Calif.

## RESULTS

*Effect of Storage.*—PTA activity was present both in serum and plasma samples with either oxalate or A-C-D solution as the anticoagulant which stored frozen at –10° to –20° C., for periods of over two years. A sample of sterile plasma stored at room temperature for over four months had normal PTA activity (Table I). Other studies have revealed that PTA activity in both normal and moderately PTA-deficient plasma increases on storage at –15° C. This is shown in Table I by the increasing PTA activity in the plasma of subject N.O. following 4 and 62 days of storage. Subject N.O. is a female with a hemorrhagic disease which was found to result from a moderate degree of PTA deficiency. This phenomenon did not occur in cases of severe PTA deficiency.

TABLE I. PTA ACTIVITY FOLLOWING STORAGE OF PLASMA

| FRACTIONS ADDED TO 1 ML. PTA DEFICIENT BLOOD | CLOTTING TIME (MIN.)* | SERUM PROTHROMBIN TIME (SEC.)† | |
|---|---|---|---|
| | | 1 HR. | 24 HR. |
| 0.1 ml. 0.85% NaCl | 17 | 15 | 26 |
| 0.1 ml. A-C-D plasma, stored 4 months at room temp. (20° to 28° C.) | 7 | 40 | 66 |
| 0.1 ml. 0.85% NaCl | 20 | 10 | |
| 0.1 ml. fresh normal plasma | 8 | 41 | |
| 0.1 ml. A-C-D plasma, frozen 1 year | 8 | 53 | |
| 0.05 ml. 0.85% NaCl | 19 | 15 | 22 |
| 0.05 ml. Patient N.O. plasma, fresh | 12 | 15 | 29 |
| 0.05 ml. 0.85% NaCl | 12 | 12 | 23 |
| 0.05 ml. N.O. plasma, frozen 4 days | 7 | 24 | 44 |
| 0.05 ml. 0.85% NaCl | 17 | 15 | 26 |
| 0.05 ml. N.O. plasma, frozen 62 days | 7 | 30 | 56 |

*Control value of the clotting time: 5 to 11 minutes.

†Control value of the serum prothrombin time at 1 hr. after coagulation: above 24 seconds; borderline 20 to 24 seconds, abnormal below 20 seconds.

*Effect of Temperature.*—PTA activity was partially decreased by exposure of normal plasma and serum to 60° C. (Table II). The exposure of rabbit brain thromboplastin to 60° C. for 20 minutes, as described by Quick,[10] produced a marked diminution in PTA activity when tested by adding 0.05 ml. heated thromboplastin to 2 ml. PTA deficient blood (4 per cent replacement). However, the addition of 0.1 ml. heated thromboplastin to 1 ml. PTA deficient blood (17 per cent replacement) produced a marked increase in prothrombin utilization (Table II).

*$BaSO_4$-Treated Plasma and Serum.*—$BaSO_4$-treated normal plasma and serum have consistently corrected the PTA deficiency (Table III). Plasma samples treated 2 times with $BaSO_4$ did not lose PTA activity. However, it was noted that Aggeler's PTC preparation,[2, 6] obtained from the citrate eluate of $BaSO_4$ following adsorption of either plasma or serum, contained PTA activity. Further studies (Table IV) have revealed that variable small amounts of PTA were adsorbed by $BaSO_4$ from normal plasma or serum, and that PTA activity could be washed from the $BaSO_4$ with 0.85 per cent NaCl and then eluted from the $BaSO_4$ by citrate. Similar studies in PTC deficiency reveal that

PTC is completely removed both from plasma and serum by $BaSO_4$. However, no PTC activity could be removed by 0.85 per cent NaCl, but was readily eluted by citrate as reported by Aggeler and associates.[2, 6]

TABLE II. EFFECTS OF TEMPERATURE ON PTA ACTIVITY

| FRACTION ADDED TO 1 ML. PTA DEFICIENT BLOOD | CLOTTING TIME (MIN.) | SERUM PROTHROMBIN TIME (SEC.) | |
|---|---|---|---|
| | | 1 HR. | 24 HR. |
| 0.1 ml. 0.85% NaCl | 13 | 11 | |
| 0.1 ml. plasma | 7 | 46 | |
| 0.1 ml. plasma exposed to 60° C. 10 min. | 9 | 23 | |
| 0.1 ml. serum | 4 | 44 | |
| 0.1 ml. serum exposed to 60° C. 10 min. | 9 | 26 | |
| 0.1 ml. 0.85% NaCl | 29 | 8 | |
| Thromboplastin,* (0.05 ml. + 2 ml. PTA blood) | 3 | 180 | |
| Thromboplastin exposed to 60° C. 20 min. (0.05 ml. + 2 ml. PTA blood) | 4 | 23 | |
| Thromboplastin exposed to 60° C. 20 min. (0.1 ml. + 1 ml. PTA blood) | 3 | 85 | |
| 0.1 ml. 0.85% NaCl | 17 | 15 | 30 |
| Thromboplastin (0.05 ml. + 2 ml. PTA blood) | 6 | 57 | 105 |
| Thromboplastin exposed to 60° C. 20 min. (0.05 ml. + 2 ml. PTA blood) | 6 | 19 | 31 |

*Prepared from Dried Rabbit Brain Thromboplastin, Difco Laboratories.

TABLE III. PTA ACTIVITY IN $BaSO_4$ ADSORBED NORMAL PLASMA AND SERUM

| FRACTION ADDED TO 1 ML. PTA DEFICIENT BLOOD | CLOTTING TIME (MIN.) | SERUM PROTHROMBIN TIME (SEC.) 1 HR. |
|---|---|---|
| 0.1 ml. 0.85% NaCl | 13 | 11 |
| 0.1 ml. plasma | 7 | 46 |
| 0.1 ml. serum | 4 | 44 |
| 0.1 ml. $BaSO_4$ plasma | 8 | 44 |
| 0.1 ml. 0.85% NaCl | 19 | 15 |
| 0.1 ml. serum | 7 | 50 |
| 0.05 ml. serum | 7 | 37 |
| 0.1 ml. $BaSO_4$ serum | 7 | 55 |
| 0.05 ml. $BaSO_4$ serum | 8 | 45 |
| 0.1 ml. $BaSO_4$ plasma | 8 | 54 |
| 0.1 0.85% NaCl | 15 | 14 |
| 0.1 plasma | 8 | 28 |
| 0.1 $BaSO_4$ plasma (adsorbed 1 time) | 8 | 32 |
| 0.1 $BaSO_4$ plasma (adsorbed 2 times) | 8 | 33 |

TABLE IV. PTA AND PTC ACTIVITIES OF $BaSO_4$ ADSORBED PLASMA AND SERUM AND THE CITRATE ELUATES OF THE $BaSO_4$

| 0.1 ML. FRACTION ADDED TO 1 ML. BLOOD | PTA DEFICIENT BLOOD | | PTC DEFICIENT BLOOD | |
|---|---|---|---|---|
| | CL. T. (MIN.) | S. P. T. (SEC.) | CL. T. (MIN.) | S. P. T. (SEC.) |
| 0.85% NaCl | 22 | 14 | 17 | 10 |
| Plasma | 10 | 20 | 8 | 18 |
| $BaSO_4$ adsorbed plasma | 11 | 21 | 17 | 14 |
| 0.85% NaCl wash of $BaSO_4$ | 12 | 16 | 17 | 13 |
| Citrate eluate of $BaSO_4$ | 9 | 22 | 8 | 62 |
| Serum | 9 | 25 | 8 | 27 |
| $BaSO_4$ adsorbed serum | 11 | 20 | 13 | 13 |
| 0.85% NaCl wash of $BaSO_4$ | 11 | 22 | 13 | 13 |
| Citrate eluate of $BaSO_4$ | 9 | 25 | 8 | 56 |

Cl. T. = Clotting time.
S. P. T. = Serum protein time.

*Seitz Filtration.*—As shown in Table V, Seitz-filtered plasma lost a small degree of PTA activity. The 50 per cent asbestos filter adsorbed slightly more PTA than did the 20 per cent filter.

TABLE V. PTA ACTIVITY IN SEITZ-FILTERED PLASMA

| 0.1 ML. FRACTION ADDED TO 1 ML. PTA DEFICIENT BLOOD | CLOTTING TIME (MIN.) | SERUM PROTHROMBIN TIME (SEC.) | |
|---|---|---|---|
| | | 1 HR. | 24 HR. |
| 0.85% NaCl | 17 | 14 | 23 |
| Plasma | 6 | 28 | 45 |
| $BaSO_4$ adsorbed plasma | 8 | 22 | 35 |
| Seitz-filtered plasma, 20% asbestos | 9 | 22 | 35 |
| Seitz-filtered plasma, 50% asbestos | 9 | 20 | 30 |

*Ammonium Sulfate Fractions.*—PTA activity was chiefly present in the plasma fraction precipitated at 25 to 33 per cent saturation with ammonium sulfate (Table VI). Minimal amounts were found in the 0 to 25 per cent fraction, and the 33 to 50 per cent fraction contained small amounts.

TABLE VI. PTA ACTIVITY IN AMMONIUM SULFATE FRACTIONS OF NORMAL PLASMA

| 0.1 ML. FRACTION ADDED TO 1 ML. PTA DEFICIENT BLOOD | CLOTTING TIME (MIN.) | SERUM PROTHROMBIN TIME (SEC.) |
|---|---|---|
| 0.85% NaCl | 13 | 11 |
| 0 to 25% ammonium sulfate saturation | 10 | 18 |
| 0 to 33% ammonium sulfate saturation | 6 | 42 |
| 0 to 50% ammonium sulfate saturation | 6 | 45 |
| 0.85% NaCl | 22 | 15 |
| 25 to 33% ammonium sulfate saturation | 8 | 39 |
| 33 to 50% ammonium sulfate saturation | 13 | 21 |

*Cohn Fractions.*—PTA was present chiefly in Fraction IV-1 (Table VII). Fractions III and I contained small amounts of PTA while none was present in Fractions II, IV-4, or V.

TABLE VII. PTA ACTIVITY IN COHN FRACTIONS

| FRACTION ADDED TO 1 ML. PTA DEFICIENT BLOOD | CONCENTRATION MG./ML. | CLOTTING TIME (MIN.) | SERUM PROTHROMBIN TIME (SEC.) | |
|---|---|---|---|---|
| | | | 1 HR. | 24 HR. |
| 0.05 ml. 0.85% NaCl | | 16 | 16 | 19 |
| 0.05 ml. Fraction I (undialyzed) | 7.6 | 12 | 20 | 23 |
| 0.05 ml. Fraction I (dialyzed | 7.6 | 13 | 19 | 23 |
| 0.05 ml. Fraction II and III (undialyzed) | 37.0 | 9 | 53 | 53 |
| 0.05 ml. Fraction II and III (dialyzed) | 37.0 | 9 | 34 | 45 |
| 0.05 ml. Fraction IV (undialyzed) | 19.0 | 13 | 24 | 25 |
| 0.05 ml. Fraction IV (dialyzed) | 19.0 | 12 | 24 | 27 |
| 0.05 ml. Fraction V (undialyzed) | 60.0 | 18 | 15 | 17 |
| 0.05 ml. Fraction V (dialyzed) | 60.0 | 16 | 19 | 19 |
| 0.1 ml. 0.85% NaCl | | 15 | 9 | 26 |
| 0.1 ml. Fraction I (undialyzed) | 7.6 | 11 | 20 | 40 |
| 0.1 ml. Fraction II (undialyzed) | 10.2 | 16 | 14 | 28 |
| 0.1 ml. Fraction III (undialyzed) | 26.8 | 6 | 23 | 39 |
| 0.1 ml. Fraction IV-1 (undialyzed) | 8.0 | 6 | 44 | 95 |
| 0.1 ml. Fraction IV-4 (undialyzed) | 10.8 | 16 | 14 | 26 |

*Electrophoresis Fractions.*—Serum fractions separated by paper electrophoresis revealed that PTA activity was present in the area between gamma globulin and beta$_1$ globulin, as shown in Fig. 1.

| FRACTION | | $\gamma$ GLOB. | (?) $\beta_2$ GLOB. | $\beta_1$ GLOB. | $\alpha_2$ GLOB. | $\alpha_1$ GLOB. | ALB. | .85% NaCl |
|---|---|---|---|---|---|---|---|---|
| CLOTTING TIME (MIN.) | | 11 | 7 | 15 | 15 | 15 | 15 | 17 |
| SERUM PRO-THROMBIN TIME (SEC.) | 1 HR. | 14 | 45 | 16 | 16 | 15 | 15 | 15 |
| | 24 HR. | 28 | 62 | 29 | 26 | 28 | 31 | 26 |
| ELECTRO-PHORETIC PATTERN | | | | | | | | |
| | | − | ORIGIN | | | | + | |

Fig. 1.—PTA activity in fractions of normal serum separated by filter paper electrophoresis. All the PTA activity was concentrated in the region between beta$_1$ and gamma globulins, and probably beta$_2$ globulin.

## DISCUSSION

These studies have demonstrated that PTA is a globulin with properties as summarized in Table VIII. For purposes of comparison, the properties of the two plasma factors closely related to PTA in the clotting mechanism, antihemophilic globulin (AHG), and plasma thromboplastin component (PTC) have been included.

PTA shows certain similarities and dissimilarities to both AHG and PTC. Common properties of PTA and PTC are: presence in serum (both unconsumed during clotting); similar distribution in Cohn fractions (chiefly present in IV-1 and III, and slight amounts in I); similar localization in serum separated by paper electrophoresis[11] (located between beta$_1$ and gamma globulin, and probably beta$_2$ globulin). PTA differs from PTC in the following aspects: ammonium sulfate fractionation (maximal PTA activity in the 25 to 33 per cent fraction, PTC activity in the 33 to 50 per cent fraction); adsorption by $BaSO_4$ treatment of plasma or serum (PTC completely removed, PTA loosely adsorbed in variable, small quantities); Seitz filtration of plasma or serum (PTC completely removed, PTA slightly removed).

PTA differs from AHG in presence in serum and distribution in ammonium sulfate and Cohn fractions. There are slight differences in the effects of $BaSO_4$ adsorption and Seitz filtration of plasma.

TABLE VIII. SUMMARY OF PROPERTIES OF PTA, PTC, AND AHG.

| FRACTION | PTA ACTIVITY | PTC ACTIVITY[2, 6, 7, 11] | AHG ACTIVITY[12-15] |
|---|---|---|---|
| Normal plasma | Present | Present | Present |
| $BaSO_4$ adsorbed normal plasma | Present | Absent | Present |
| Normal serum | Present | Present | Absent |
| $BaSO_4$ adsorbed normal serum | Present | Absent | Absent |
| 0.85% NaCl wash of $BaSO_4$ after adsorption of either normal serum or plasma | Slight to moderate | Absent | None to slight |
| Citrate eluate of $BaSO_4$ after NaCl wash | Slight to moderate | Marked | None to slight |
| Ammonium sulfate fraction of plasma, maximal activity | 25 to 33% | 33 to 50% | 0 to 25% |
| Seitz-filtered normal plasma | Slight to moderate decrease | Absent | Present |
| Cohn fractions (maximal activity) | IV-1 and III | IV-1 and III | I |
| Stored normal plasma | Present | Present | Disappears |
| Heated rabbit brain thromboplastin[10] | Diminished | Diminished | Diminished |
| Electrophoresis of normal serum, filter paper technique | $Beta_2$ glob. (between $beta_1$ and gamma) | $Beta_2$ glob. (between $beta_1$ and gamma) | |

The apparent increase in PTA activity in stored plasma samples obtained from subjects with hemorrhagic disease caused by moderate degrees of PTA deficiency is of particular interest. Although the mechanism is not known, there are currently two possible explanations: (1) Clotting reactions may occur at a slow rate in the frozen sample which will compensate for the PTA deficiency and hence will correct the deficiency in the test system. (2) Inhibitor substances disappear on storage. From a practical standpoint, this phenomenon provides difficulty in interpreting the matching studies performed on frozen or stored samples sent great distances from one laboratory to another. Analysis of the properties of the deficient clotting factor may provide a diagnosis of a deficient plasma thromboplastin factor in the event that reliable matching studies cannot be obtained.

## SUMMARY

Studies on various properties of plasma thromboplastin antecedent (PTA) have revealed that it is a globulin, maximally precipitated at 25 to 33 per cent ammonium sulfate saturation of normal plasma, stable on storage at temperatures ranging from –20° to 28° C., chiefly present in Cohn fraction IV-1, and localized in the $beta_2$ globulin (between gamma and $beta_1$ globulin) fraction of serum separated by paper electrophoresis. PTA activity is present in normal serum. Seitz filtration and $BaSO_4$ treatment of normal plasma and serum irregularly remove slight amounts of PTA which can be eluted both by sodium chloride and sodium citrate.

## REFERENCES

1. Rosenthal, R. L., Dreskin, O. H., and Rosenthal, N.: New Hemophilia-Like Disease Caused by Deficiency of a Third Plasma Thromboplastin Factor, Proc. Soc. Exper. Biol. & Med. **82**: 171, 1953.
2. Aggeler, P. M., White, S. G., Glendening, M. B., Page, E. W., Leake, T. B., and Bates, G.: Plasma Thromboplastin Component (PTC) Deficiency: A New Disease Resembling Hemophilia, Proc. Soc. Exper. Biol. & Med. **79**: 692, 1952.

3. Rosenthal, R. L.: Hemophilia and Hemophilia-Like Diseases Caused by Deficiencies in Plasma Thromboplastin Factors: Anti-Hemophilic Globulin (AHG), Plasma Thromboplastin Component (PTC) and Plasma Thromboplastin Antecedent (PTA), Am. J. Med. **17**: 57, 1954.
4. Rosenfield, R. E., and Tuft, H. S.: Estimation of Prothrombin Level From Prothrombin Time, Am. J. Clin. Path. **17**: 405, 1947.
5. Alexander, B., Goldstein, R., and Landwehr, G.: The Prothrombin Conversion Accelerator of Serum (SPCA): Its Partial Purification and Its Properties Compared With Serum Ac-Globulin, J. Clin. Invest. **29**: 881, 1950.
6. White, S. G., Aggeler, P. M., and Glendening, M. B.: Plasma Thromboplastin Component (PTC); A Hitherto Unrecognized Blood Coagulation Factor; Case Report of PTC Deficiency, Blood **8**: 101, 1953.
7. White, S. G., Aggeler, P. M., and Emery, B. E.: Plasma Thromboplastin Component (PTC) Potency of Plasma Fractions, Proc. Soc. Exper. Biol. & Med. **83**: 69, 1953.
8. Durrum, E. L.: A Microelectrophoretic and Microionophoretic Technique, J. Am. Chem. Soc. **72**: 2943, 1950.
9. Kunkel, H. G., and Tiselius, A.: Electrophoresis of Proteins on Filter Paper, J. Gen. Physiol. **35**: 89, 1951.
10. Quick, A. J., Stopp, W. F., and Hussey, M. S.: The Effect of Heating on Thromboplastin Activity of Rabbit Brain Extract. A New Test for the Diagnosis of Hemophilia, J. LAB. & CLIN. MED. **39**: 142, 1952.
11. Aggeler, P. M., Spaet, T. H., and Emery, B. E.: Purification of Plasma Thromboplastin Factor B (Plasma Thromboplastin Component) and Its Identification as a Beta$_2$ Globulin, Science **119**: 806, 1954.
12. Lozner, E. L., and Taylor, F. H. L.: The Coagulation Defect in Hemophilia; Studies of the Clot-Promoting Activity Associated With Plasma Euglobulin in Hemophilia, J. Clin. Invest. **18**: 821, 1939.
13. Taylor, F. H. L., Davidson, C. S., Tagnon, H. J., Adams, M. A., MacDonald, A. H., and Minot, G. R.: Studies in Blood Coagulation. The Coagulation Properties of Certain Globulin Fractions of Normal Human Plasma In Vitro, J. Clin. Invest. **24**: 698, 1945.
14. Graham, J. B., Buckwalter, J. A., Hartley, L. J., and Brinkhous, K. M.: Canine Hemophilia. Observations on the Course, the Clotting Anomaly, and the Effects of Blood Transfusions, J. Exper. Med. **90**: 97, 1949.
15. Langdell, R. D., Wagner, R. H., and Brinkhous, K. M.: Effect of Antihemophilic Factor on One-Stage Clotting Tests, J. LAB. & CLIN. MED. **41**: 637, 1953.

# PERFUSION STUDIES OF THE ISOLATED YOUNG RAT LIVER

DAVID L. GUNBERG,* PH.D., WILLIAM R. LYONS, M.D., PH.D., AND
RUTH E. JOHNSON, A.B.
BERKELEY, CALIF.

MAINTENANCE of isolated organs by perfusion is a venerable technique.[1] Apparatus described in the past, however, were usually adapted to large organs.[2] Recently two machines have been described which are suitable for investigations of the in vitro function of organs taken from small laboratory animals.[3, 4] The apparatus described by Long and Lyons[4] was designed for experiments on isolated small organs. In order to compare this relatively new perfusion apparatus with an established machine, the liver was chosen as the experimental organ. This permitted some comparison of results with those previously reported by Brauer and associates.[3]

The following experiments, therefore, were primarily concerned with the development of a perfusate and an examination of the adequacy of the small perfusion apparatus in the maintenance of function and histologic integrity in young isolated rat livers.

## MATERIALS AND METHODS

*Perfusion Medium.*—The medium reported here is the result of numerous unreported experiments and, in the opinion of these authors, provided the best results.

25 ml. whole heparinized blood.
75 ml. White's media[5] (170 mg. per cent glucose).
5 mg. sodium heparin (at start of run and every two hours).
8 mg. potassium penicillin (at start of run and every two hours).
20 mg. dihydrostreptomycin sulfate (at start of run and every two hours).

*Organs.*—Livers were obtained from ether-anesthetized, starved (16 to 20 hours) female rats of the Long-Evans strain (21 to 30 days of age). The portal vein was initially dissected free and 0.5 mg. of sodium heparin was administered. The bile duct was then isolated and severed close to the duodenum. Prior to removal of the organ one of the small lobes of the liver was ligated and removed for a control. Removal of the organ was accomplished by dissecting the diaphragm free from its attachments, resecting in a way that excluded the esophagus. As a final step the portal vein, hepatic artery, and inferior vena cava were severed. The organ was quickly transferred to the perfusion chamber where the portal vein and bile duct were cannulated. The latter was connected with the system of tubing normally used in a "closed system" (see Long and Lyons[4]) for venous return to the reservoir. This system of tubing was attached to a long piece of calibrated capillary polyethylene tubing on the exterior of the perfusion apparatus, thus permitting easy observation of bile production.

*Perfusion.*—The organ was initially flushed with White's media and this fluid was then removed from the perfusion chamber. With practice, the length of time during which the liver was deprived of a circulation was reduced to a maximum of two minutes. Bile volume, perfusate pressure at the portal vein, and perfusate flow rate through the organ

From the Department of Anatomy, School of Medicine, University of California, Berkeley, California.
Received for publication Aug. 12, 1954.
*Present address: Department of Anatomy, School of Medicine, University of Southern California, Los Angeles, Calif.

were determined every half hour. Biopsies were taken at the end of four hours, eight hours, and, in one instance, twenty-seven hours. These tissues were fixed in Carnoy's fluid and stained with the periodic acid-Schiff's reagent technique; appropriate adjacent sections were digested with saliva in order to analyze for histologically evident glycogen deposition.

## RESULTS

Average bile flow during the first four hours in the perfused livers is summarized in the graph presented in Fig. 1. These rates are adjusted to liver weights and indicate the flow per gram of liver tissue. Seven of the eight preparations were allowed to continue after a biopsy was taken four hours after initiation of the run. At the eight-hour interval these organs were producing an average bile flow of 0.5 $\mu$l per minute per gram (0.03-1.0). It was incidentally noted that the addition of thyroxin to the perfusate significantly increased bile flow.

At constant portal vein pressure of 20 mm. Hg, perfusate flow through the organ consistently revealed a sharp increase during the first hour of perfusion (Fig. 2). After this initial rise the perfusate-flow rate became relatively constant although a gradual increase was noted. A positive correlation between flow rate and bile production could not be demonstrated.

Maintenance of histologic integrity was exceptionally good in the four-hour biopsies, and some of the organs perfused for longer periods revealed morphologically normal hepatic tissue for periods up to twenty-seven hours (Fig. 3). In no instance, however, was there an increase in histologically evident glycogen, but rather in many instances an observable decrease when compared with control sections. The addition of insulin, cortisone, and/or thyroxin to the perfusate, singularly or in combination, did not alter these results. Similarly an increase of perfusate sugar levels to a maximum of 450 mg. per cent also failed to produce a deposition of glycogen as determined in the histologic preparations of these young rat livers.

## DISCUSSION

The data presented demonstrate that in vitro function, as determined by bile flow and histologic integrity, can be accomplished in young isolated rat livers maintained on the Long-Lyons perfusion apparatus. Survival periods in excess of four hours with a duration of as much as twenty-seven hours, were noted. Contrary to the report of Brauer and associates[3] on older rats, starvation of our young donors prior to perfusion did not appear to alter the results. Also, the duration of function in these isolated young livers exceeded that which might be anticipated from former reports.[3]

A rapid increase in perfusate flow during the first hour of perfusion indicates a possible vasodilatation. Brauer and co-workers[6] have recently described a vasoconstrictor present in whole heparinized blood, believed to be responsible for 50 to 60 per cent of the vasomotor tone in the liver in situ. The destruction or loss through dialysis of such a substance during the course of perfusion would explain the sudden rise in perfusate-flow rates.

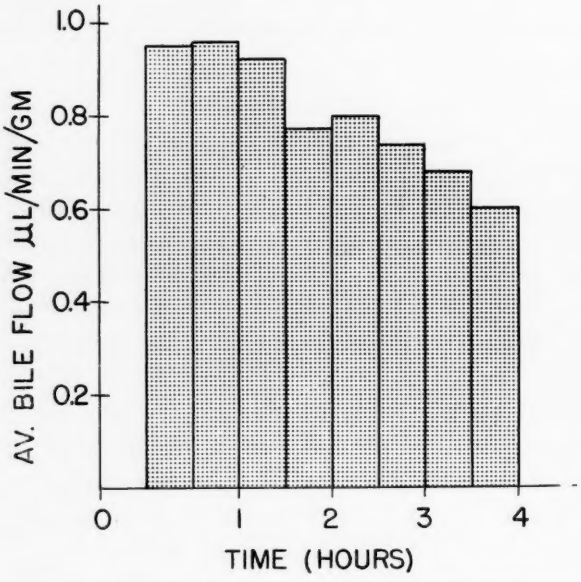



Fig. 1.—Bile flow in perfused livers during the first four hours.

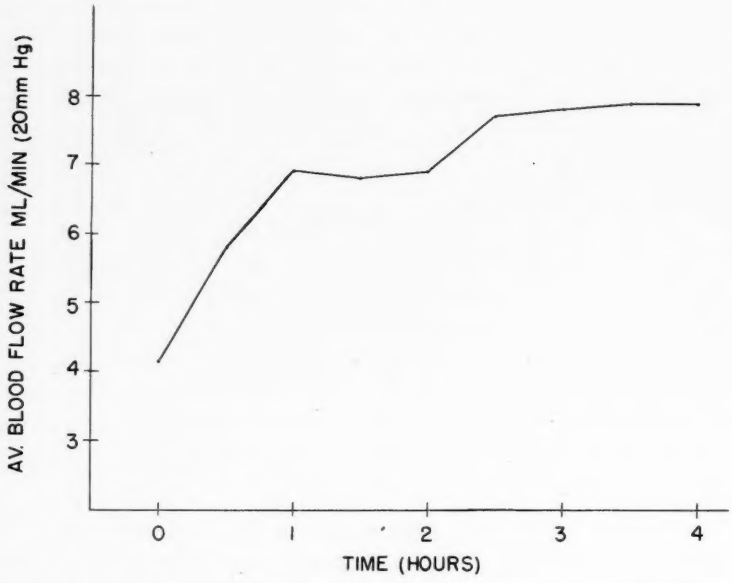



Fig. 2.—Blood flow in perfused livers at constant portal vein pressure.

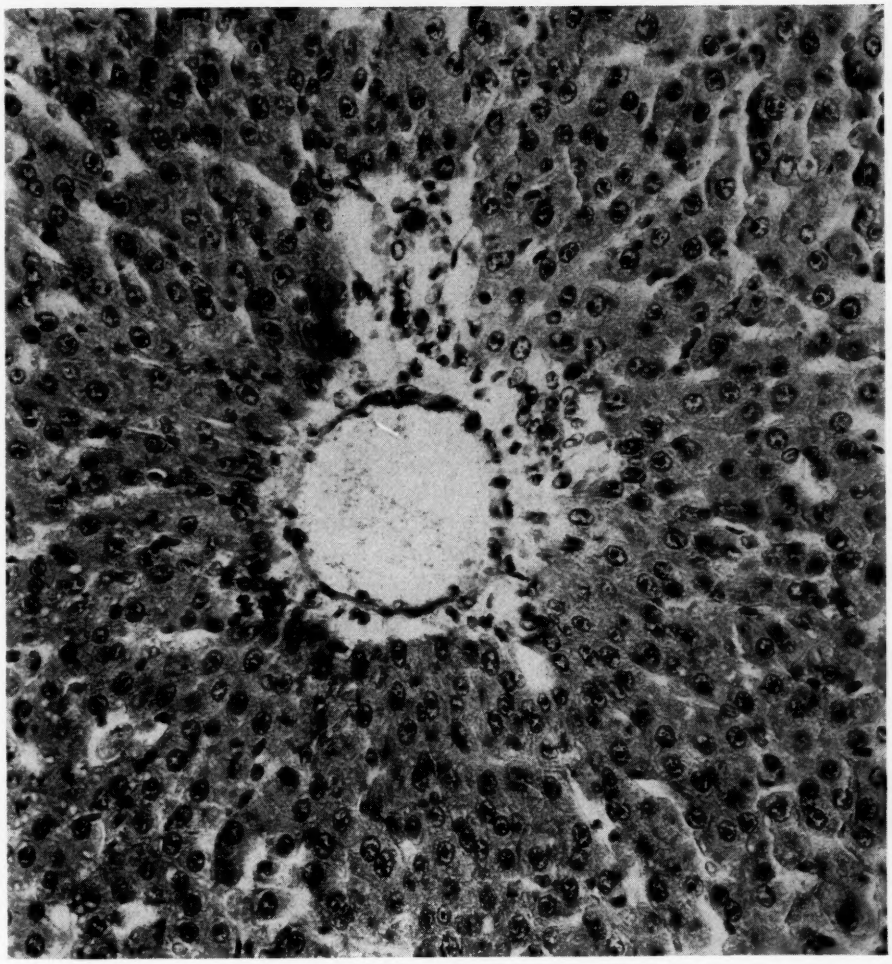


Fig. 3.—Liver of 60 Gm. female rat maintained on the perfusion apparatus for twenty-seven hours. This periportal area shows well-preserved hepatic cells with little or no dilatation of the sinusoids. Some evidence of edema may be represented by the clear perivascular area. (×340.)

Lack of glycogen deposition in the perfused young rat liver, in the presence of adequate levels of circulating glucose in whole blood and White's media, indicates that this combined perfusion mixture either lacks other important ingredients or loses them through dialysis.

## SUMMARY AND CONCLUSION

It has been demonstrated that the small Long-Lyons plastic perfusion apparatus when employed in the manner described functions as well as other larger models employed in the maintenance of isolated rat livers.

We are most grateful to Wayne B. Bigelow for his technical assistance during the early phases of the above experiments.

## REFERENCES

1. Brodie, T. G.: The Perfusion of Surviving Organs, J. Physiol. **29**: 26, 1903.
2. Carrel, A., and Lindbergh, C. A.: The Culture of Organs, Hamish Hamilton Medical Books, London, 1938.
3. Brauer, R. W., Pessotti, R. L., and Pizzolato, P.: Isolated Rat Liver Preparation—Bile Production and Other Basic Properties, Proc. Soc. Exper. Biol. & Med. **78**: 174, 1951.
4. Long, L. A., and Lyons, W. R.: A Small Perfusion Apparatus for the Study of Surviving, Isolated Organs, J. LAB. & CLIN. MED. **44**: 614, 1954.
5. White, P. R.: Cultivation of Animal Tissues in Vitro in Nutrients of Precisely Known Constitution, Growth **10**: 231, 1946.
6. Brauer, R. W., Leong, G. F., Holloway, R. S., Krebs, J. S., and Pessotti, R. L.: Isolated Perfused Rat Liver—Changes in Physiological State Associated With Transfer From Animal to Perfusion System, J. Pharmacol. & Exper. Therap. **110**: 7, 1954.

# IN VITRO STUDIES OF RED CELL FRAGILITY IN ANTI-D HEMOLYTIC DISEASE OF THE NEWBORN

TIBOR J. GREENWALT, M.D.,* AND DEMETRIOS C. TRIANTAPHYLLOPOULOS, M.D.**
MILWAUKEE, WIS.

WITH THE TECHNICAL ASSISTANCE OF MAVIS MOORMAN

THE severity of hemolytic disease of the newborn resulting from maternal anti-D ($Rh_0$) isoimmunization shows no regular correlation with the level of the prenatal antibody titer or the degree of "coating" of the fetal red blood cells.[1] Unknown fetal factors may be concerned in influencing the rate of the hemolytic mechanism. The level of complement apparently does not play a determining role.[2] The rate of red cell destruction is not the sole factor in determining clinical severity. Though managed similarly an infant with severe anemia and pronounced erythroblastosis may do better than one with little or no anemia. Variation in the ability of the liver to dispose of the end products of red cell breakdown partly explains this apparently paradoxical situation.

The osmotic and mechanical fragilities of the red blood cells during the early newborn period have not been extensively studied. The hypotonic fragility of the red blood cells in normal newborn infants has been reported to be the same as that of healthy adults by some investigators,[3, 4] but as decreased[5] or increased by others.[6] The mechanical fragility of the erythrocytes in the neonatal period has been found to be greater than that of adults.[7] Bongiovanni and Abelson[5] observed increased mechanical and hypotonic fragilities in infants with erythroblastosis fetalis.

We have studied the hypotonic and mechanical fragilities of the red cells before and after incubation in a series of normal infants and infants with proved hemolytic disease due to anti-D.

## MATERIALS AND METHODS

*Blood Samples.*—The blood specimens consisted of cord blood or the first specimen withdrawn at the time of replacement transfusion. Dry double oxalate mixture was the anticoagulant used. Care was taken to avoid unnecessary contamination during all the procedures but no attempt was made to insure or to test for the presence of sterility. The blood from healthy donors was collected in A. C. D. solution as part of another study.

*Determination of Hypotonic Saline Fragility.*—The simplified test described by Suess and associates[8] was employed with minor modifications. Seven tubes with 0.80, 0.56, 0.48, 0.44, 0.40, and 0.32 per cent sodium chloride solution, and distilled water were used. The stock solutions were prepared with freshly distilled water and C.P. sodium chloride. The saline concentrations of the solutions were checked by the silver nitrate method. The pH values of the individual solutions were not determined. The distilled water used has a fairly constant pH value of 6.8. Five milliliters of each solution was pipetted into cuvettes. The cuvettes were 13 by 100 mm. test tubes calibrated for use with a specially constructed

Received for publication Aug. 14, 1954.

*Medical Director, Milwaukee Blood Center, Milwaukee, Wis.; Assistant Clinical Professor of Medicine, Marquette University School of Medicine, Milwaukee, Wis.

**Research Associate, Milwaukee Blood Center, Milwaukee, Wis.

adapter in the Coleman junior spectrophotometer. The blood specimen was inverted several times to insure uniform mixing and oxygenation. Ten cubic millimeters of oxalated whole blood was pipetted into each tube using a 0.2 ml. pipette calibrated at 0.001 ml. in intervals. Each tube was inverted three times immediately after the addition of the blood to insure complete mixing and to avoid drying of the blood on the wall of the tube. After standing at room temperature for one hour the tubes were centrifuged at 2,000 r.p.m. for 15 minutes. The hemoglobin concentration in the supernatant was read directly in each tube at 545 m$\mu$, using the 0.80 per cent tube as the blank. The density readings were used to calculate the per cent hemolysis in each tube.

The incubated hypotonic fragility tests were performed in the same manner on an aliquot of the original specimen which had been kept in a 37° C. water bath for 24 hours. The tubes were sealed with Parafilm* to prevent evaporation and contamination with the water of condensation.

The 50 per cent hemolysis values were determined as described by Parpart and associates.[9]

*Mechanical Fragility.*—Mechanical fragilities were determined by a method adapted for use in this laboratory. It is a variation of the method described by Shen and associates.[10] The hematocrit of each specimen was adjusted to approximately 40 per cent. After gentle but complete mixing the hemoglobin concentration (c) was determined by the oxyhemoglobin method in a Coleman junior spectrophotometer at 545 m$\mu$. This was accomplished by adding 0.02 ml. of the specimen to 5 ml. of 0.1 per cent sodium carbonate. Another 0.02 ml. portion was pipetted into 5 ml. of 1.25 per cent sodium chloride. After mixing the latter was centrifuged and the supernatant transferred to a 12 by 75 mm. Coleman cuvette containing 0.02 ml. of 25 per cent sodium carbonate to bring the concentration of sodium carbonate to 0.1 per cent. The hemoglobin concentration (a) in the original plasma was thus determined. Two milliliters of the adjusted blood specimen was placed in a 13 by 100 mm. tube with twenty 4 mm. glass beads. The tube was sealed with a 00 rubber stopper covered with Parafilm and placed in a Bryan-Garrey pipette rotor† for one hour at room temperature at 60 r.p.m. At the end of this period 0.02 ml. of the sample was pipetted into 5 ml. of 1.25 per cent sodium chloride. After inversion of the tube three times it was centrifuged and the crystal-clear supernatant was transferred to a cuvette containing 0.02 ml. of 25 per cent sodium carbonate. The hemoglobin content (b) was determined.

The mechanical fragility was determined by the formula given by Shen and co-workers:[10]

$$\text{M.F. per cent} = \frac{(b-a)\,100}{c-a}$$

## RESULTS

The hypotonic fragility of the red blood cells in cord or venous blood was determined in 16 normal newborn infants before and after incubating the blood for 24 hours at 37° C. The results are graphically illustrated in Fig. 1, which was constructed by calculating the mean per cent hemolysis in each concentration of saline used. For comparison the average values for the unincubated blood of 24 adults are plotted. The fragility curve of fetal blood is shifted to the left after incubation, indicating increase in fragility. The results were also analyzed by computing the mean corpuscular fragility (the concentration of saline in which 50 per cent of the corpuscular hemoglobin is released) for each subject according to the method of Parpart and associates[9] (Table I). The mean corpuscular fragility for the group was 0.426 per cent sodium chloride prior to incubation and 0.499 per cent sodium chloride after incubation. The mean fragility for the 24 adults was found to be 0.464 per cent sodium chloride.

*Manufactured by Marathon Corporation, Menasha, Wis.
†A. S. Aloe Co., Catalog No. 10870.

The mean per cent hemolysis values obtained in each concentration of saline in 34 cases of hemolytic disease due to anti-D are also charted in Fig. 1. In 28 of these cases the incubated hypotonic fragility test was also performed. The nonincubated curve is shifted to the left in relation to the curve for the normal infants, indicating somewhat increased fragility. The incubated hypotonic fragility curve is asymmetric and crosses the preincubated curve. It is not as far to the left as the curve for the incubated fragility tests of the normal

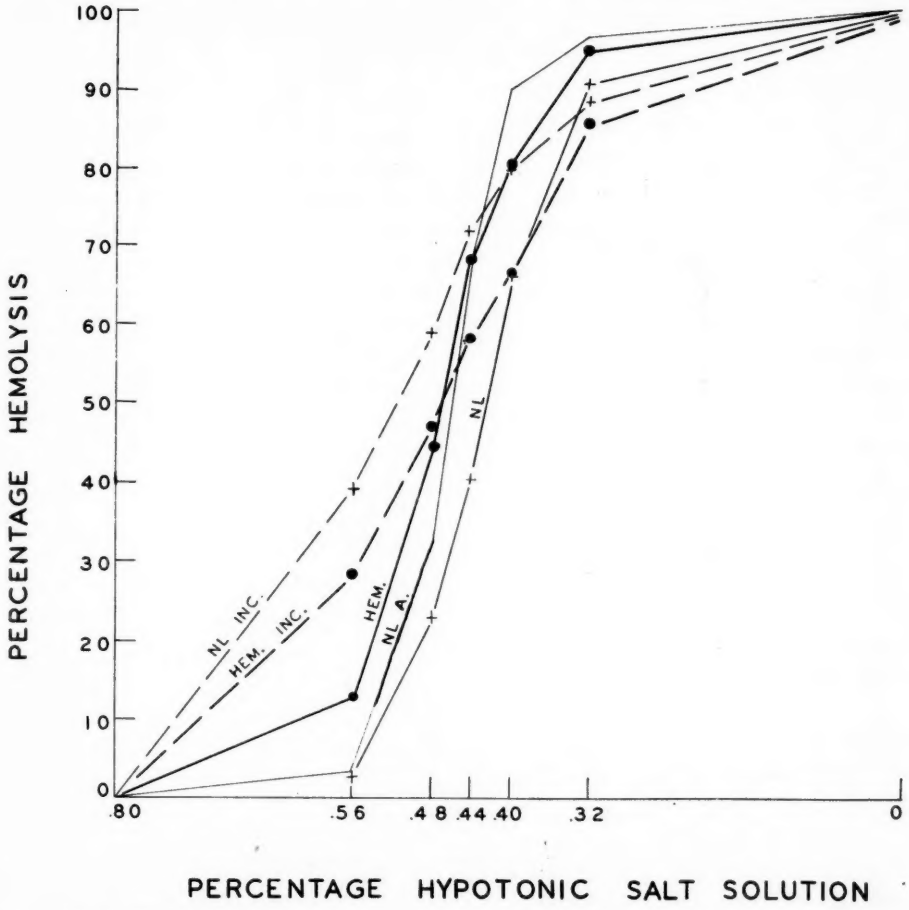



Fig. 1.—Hypotonic fragility curves. NL A.—Normal adults; NL—normal infants before incubation; NL INC.—normal infants after incubation; HEM.—hemolytic infants before incubation; HEM. INC.—hemolytic infants after incubation. Note the manner in which the postincubation curve crosses the preincubation curve.

group. These results resemble very closely those reported by Wright and coworkers[11] for idiopathic acquired hemolytic anemia. The lower end of the curve for the incubated fragilities is shifted to the left, indicating increased fragility in the higher concentrations of saline. The upper end of the curve is shifted to the right, indicating increased resistance in the lower concentrations of saline.

The average of the preincubated mean corpuscular fragilities for the hemolytic group was 0.486 per cent sodium chloride, which is significantly greater than the 0.426 per cent value for the normal group (Table I). The postin-

TABLE I. SUMMARY OF MEAN CORPUSCULAR FRAGILITY STUDIES

| | BEFORE INCUBATION | | | | | AFTER INCUBATION | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | NO. | MEAN | RANGE | STANDARD DEVIATION | P | NO. | MEAN | RANGE | STANDARD DEVIATION | P |
| Normal newborn | 16 | 0.426 | 0.360-0.485 | 0.032 | <0.01 | 16 | 0.499 | 0.395-0.650 | 0.064 | 0.05-0.01 |
| Hemolytic newborn | 34 | 0.486 | 0.400-0.640 | 0.060 | | 28 | 0.457 | 0.355-0.540 | 0.052 | |

TABLE II. SUMMARY OF MECHANICAL FRAGILITY STUDIES

| | NO. | MEAN | RANGE | STANDARD DEVIATION | P |
|---|---|---|---|---|---|
| Normal adults | 16 | 3.8 | 2.1- 6.3 | 1.1 | <0.01 |
| Normal newborn | 31 | 6.4 | 2.9-11.6 | 2.3 | >0.5 |
| Hemolytic newborn | 31 | 6.5 | 1.4-21.2 | 4.1 | |

cubated mean corpuscular fragility for the hemolytic cases was 0.457 per cent sodium chloride. This indicates a decrease in the postincubation red cell fragility in hemolytic disease due to anti-D.

The results of the mechanical fragility studies are shown in Table II. There is no significant difference between the normal and hemolytic groups. The normal newborn infants have significantly higher values than the adults.

No correlation was noted between the values for osmotic fragility and those for mechanical fragility.

## DISCUSSION

Goldbloom and Gottlieb[6] found that the red cells of normal newborn infants are less resistant to hypotonic saline than those of normal adults. Later Whitby and Hynes[3] and Adams and Cunningham[4] were unable to substantiate this observation. Our findings indicate that the red blood cells of normal newborn infants are slightly more resistant to osmotic hemolysis than those of adults. This difference can be explained, in part, by the presence of a larger percentage of reticulocytes in the blood of the newborn.[8] In agreement with Bongiovanni and Abelson[5] we find the red cells of infants with anti-D hemolytic disease to be less resistant than normals. This decrease in resistance is not related to the presence of spherocytosis which was demonstrable in only two of the 34 subjects.

The mean corpuscular fragility of the erythrocytes of the normal newborn infants increased after incubation whereas the value of the hemolytic group decreased. Plotting the values does bring out an important feature which is not revealed by the 50 per cent hemolysis values. The manner in which the postincubation curve crosses over the preincubation curve in the hemolytic infants is similar to that reported by Wright and associates[11] in idiopathic acquired hemolytic anemias which appear later in life. Hemolytic disease of the newborn resulting from isoimmunization of the mother resembles these acquired hemolytic anemias in many respects.

Our studies indicate that the results of hypotonic fragility tests performed on blood which has been incubated at 37° C. should be interpreted with a great deal of caution. In this procedure one performs the so-called "heat resistance" or the "incubation" fragility (autohemolysis) test described by Dacie[12] prior to testing osmotic fragility. Under these conditions the more "susceptible" red blood cells are broken down during the period of preincubation leaving only the more resistant population. When the hypotonic fragility test is performed on this sample in any condition with an abnormal "heat resistance" test, the red cells may appear to be more resistant to hypotonic breakdown after incubation than they were before incubation. This is certainly the case if the degree of hemolysis is determined by comparison to the 0.80 per cent sodium chloride tube set at 100 per cent transmission. If the readings are made against distilled water set at 100 per cent transmission, the amount of hemolysis which occurred during incubation is demonstrated but the readings of the hypotonic fragility test become meaningless.

The mechanical fragility was determined by a method adapted for use in our laboratory. It is important to standardize modifications of the test orig-

inally described by Shen and co-workers[10] for each laboratory. Our results for normal newborn infants and adults agree reasonably well with those previously published.[7] The mechanical fragility of the red cells of newborn infants is greater than that of adults. This can, in part, be explained by the presence of macrocytosis.[10] The mechanical fragilities of the red cells of infants with anti-D hemolytic disease do not differ significantly from those of normal newborn infants.

Correlation of the results of the hypotonic fragility and mechanical fragility studies with clinical severity in anti-D hemolytic disease of the newborn is difficult. Study of our records indicates that there is no relationship between hypotonic fragility and clinical severity. The values for mechanical fragility for infants with hemolytic disease were in the same range as those for normal newborn infants.

## SUMMARY

1. Hypotonic saline fragility studies were performed with the red cells of normal newborn infants before and after incubation at 37° C. for 24 hours. The erythrocytes of a group of infants with hemolytic disease due to anti-D were studied in an identical manner.

The hypotonic red cell fragility in infants with anti-D hemolytic disease was found to be significantly greater than that of normal infants. After incubation the fragility of the normal group increased. The fragility curve of the hemolytic infants became more skew and crossed the preincubation curve.

2. The erythrocyte mechanical fragilities were determined in normal infants, normal adults, and infants with anti-D hemolytic disease. The red blood cells of normal infants were found to have a greater mechanical fragility than those of adults. No difference was demonstrated between the normal and hemolytic groups of infants.

## REFERENCES

1. Greenwalt, T. J., and Wagner, J.: Evaluation of the Quantitative Direct Antiglobulin Test in Hemolytic Disease of the Newborn Due to Anti-D, Blood, In press.
2. Triantaphyllopoulos, D., and Greenwalt, T. J.: Complement in Hemolytic Disease of the Newborn, Blood **9**: 505, 1954.
3. Whitby, L. E. H., and Hynes, M.: The Quantitative Estimation of the Fragility of the Red Corpuscles, J. Path. & Bact. **40**: 219, 1935.
4. Adams, F. H., and Cunningham, S. C.: Fragility of Red Blood Cells from Newborn Infants and Children with Cyanotic Congenital Heart Disease, J. Pediat. **39**: 180, 1951.
5. Bongiovanni, A. M., and Abelson, N. M.: Fragility of Erythrocytes Exposed to Rh Antibodies in Vivo and in Vitro, Am. J. Dis. Child. **84**: 474, 1952.
6. Goldbloom, A., and Gottlieb, R.: Icterus Neonatorum, Am. J. Dis. Child. **38**: 57, 1929.
7. Goldbloom, R. B., Fischer, E., Reinhold, J., and Yi-Yung Hsia, D.: Studies on the Mechanical Fragility of Erythrocytes. I. Normal Values for Infants and Children, Blood **8**: 165, 1953.
8. Suess, J., Limentani, D., Dameshek, W., and Dolloff, M. J.: A Quantitative Method for the Determination and Charting of the Erythrocyte Hypotonic Fragility, Blood **3**: 1290, 1948.
9. Parpart, A. K., Loung, P. B., Parpart, E. K., Gregy, J. K., and Chase, A. I.: The Osmotic Resistance (Fragility) of Human Red Cells, J. Clin. Invest. **26**: 636, 1947.
10. Shen, S. C., Castle, W. B., and Fleming, E. M.: Experimental and Clinical Observations on Increased Mechanical Fragility of Erythrocytes, Science **100**: 387, 1944.
11. Wright, C. S., Sundharagiati, B., Bass, J. A., and Bunner, A. E.: Review of the 1952 Hematology Literature, Arch. Int. Med. **92**: 3, 1953.
12. Dacie, J. V.: Practical Hematology, Brooklyn, N. Y., 1951, Chemical Publishing Company, Inc.

# *LABORATORY METHODS*

## AN "ANTI-INFECTION CANNULA" FOR SYRINGES TO PREVENT TRANSMISSION OF INFECTIONS IN MASS IMMUNIZATION CAMPAIGNS

K. H. Gan, M.D.
Djakarta, Indonesia

With the Technical Assistance of Tjioe Swie Bie and R. Soekardi Atmadja

THE risk of transferring infections via the syringe has become a problem of primary importance, especially since Findlay and MacCallum[2] in 1937 showed that serum hepatitis may be transmitted by insufficiently sterilized syringes and needles. Contamination of a syringe used for multiple injections may be due to various factors, such as increased tissue pressure following injection, negative pressure between the needle and syringe arising during the removal of the needle from the syringe, resulting in liquid being drawn back from the needle into the syringe,[6] or small withdrawal movements of the plunger following injection.[1] Change of needles, with brief immersion of the used needles in hot paraffin (Fleming and Ogilvie, cited by Gispen),[4] or in hot glycerin[5] has not proved sufficient to prevent contamination of syringe contents. The "one-way-valve" of Gispen[4] is sound in principle, in that it is intended to prevent the backflow of liquid from the needle into the syringe, but the effect of frequent boiling on the efficiency of the soft valve remains to be determined. It is generally held that the only safe course is to use a separate syringe and needle for each person being injected.

The following is a report of an experimental study to determine in what way the risk of contaminating syringe contents may be prevented when one syringe is employed for multiple injections. It relates specifically to the effectiveness of a specially designed cannula which is attached to such a syringe.

### MATERIALS AND METHODS

The cannula employed was made from chromium-plated or nickel-plated brass and is 35 mm. long and 5 mm. broad, with a narrow channel at the distal end joining the needle and a wider channel at the proximal end joining the syringe (Fig. 1). Cannulas of different capacities were tested, namely, those having a capacity of either 0.5, 0.2, 0.15, 0.1, or 0.075 ml. In all tests care was taken to see that both needle and syringe fit well onto the cannula. This was checked before sterilization as follows: The syringe with the cannula

From the Institute for Bacteriology "Lembaga Wilhelmina," University of Indonesia, Djakarta, Indonesia.
Received for publication March 18, 1954.

TABLE I. CONTAMINATION OF THE SYRINGE CONTENTS USING "ANTI-INFECTION CANNULAS" OF VARIOUS DIMENSIONS

| EXPERIMENT NO. | TEST BACTERIUM | CAPACITY OF CANNULA | ROUTE OF INJECTION AND ANIMAL | DOSE AND OTHER PARTICULARS (SEE FOOTNOTE AND DESCRIPTION UNDER "METHODS") | WITH CANNULA | | | WITHOUT CANNULA | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | NUMBER OF TESTS | NUMBER OF CONTAMINATIONS (%) | | NUMBER OF TESTS | NUMBER OF CONTAMINATIONS (%) | |
| | | | | | | CONTENTS SYRINGE | CONTENTS NEEDLE | | CONTENTS SYRINGE | CONTENTS NEEDLE |
| 1 | *S. marcescens* | 0.2 ml. and 0.5 ml. | Subcutaneous; monkey | 0.5 ml. to 5 ml.* | 150 | 0 | 90 | 250 | 58 | 92 |
| 2 | *S. marcescens* | 0.15 ml. | Intraveneous (ear vein of rabbits) | 2 ml. | 25 | 0 | 75 | 50 | 44 | 90 |
| 3 | *S. marcescens* | 0.15 ml. | Subcutaneous; monkey | Same as test 1, but 1.5 to 5 ml. | 1,150 | 0 | 96 | 320 | 34 | 95 |
| 4 | *V. El Tor.* | 0.15 ml. | Subcutaneous; monkey | Same as test 1 | 50 | 0 | 90 | 20 | 40 | 90 |
| 5 | *S. marcescens* | 0.1 ml. | Subcutaneous; monkey | Same as test 1, but 1 to 5 ml. | 150 | 0.7 | 95 | 40 | 47 | 80 |
| 6 | *S. marcescens* | 0.1 ml. (0.075 ml.) | Subcutaneous; monkey | Like test 1, injected liquid 0.5 to 2 ml. | 124 | 4.7 | 94 | 60 | 16.6 | 92 |
| 7 | *S. marcescens* | 0.15 ml. | Subcutaneous; monkey | 3 to 4 ml. liquid injected† | 100 | 1 | 100 | 50 | 60 | 100 |
| 8 | *S. marcescens* | 0.15 ml. | Subcutaneous; monkey | Like test 7 but 1.5 to 5 ml.‡ | 39 | 0 | 97 | 102 | 81 | 97 |
| 9 | *S. marcescens* | 0.15 ml. | Intracutaneous | Like test 7, but 0.1 to 0.2 ml. | 95 | 0 | 89 | 64 | 48 | 91 |
| 10 | *S. marcescens* | 0.15 ml. | Subcutaneous | Each syringe for 5 injections of 1 ml. liquid each; performed like test 3 | 20 | 0 | 93 | 10 | 60 | 90 |
| 11 | *S. marcescens* | 0.15 ml. | Intramuscular | The same as test 3, 3 to 4 ml. | 50 | 0 | 90 | 68 | 28 | 95 |

*Needle after injection of the liquid pressed deeper into the tissue; afterward syringe loosened from needle-cannula assembly.
†Needle after injection not pressed deeper into the tissue.
‡Needle-cannula assembly loosened from the syringe only after pulling out from tissue.

attached was filled with 1 to 2 ml. of water, and after removal of air from the cannula-syringe assembly, the distal opening of the cannula was closed with a finger and the plunger was pressed forward vigorously for five seconds, to make sure that no leakage occurred from the connection between the cannula and syringe. Leakage from the cannula-needle junction can be detected easily during the injection of the liquid into the tissue.

The cannula (Fig. 2) was employed with 2 and 5 ml. graduated syringes ("Record" syringes with a metal tip and a metal plunger or all glass syringes with a metal tip), and with a No. 14 or finer needle (Fig. 3). Before and after use in a test, the assembly was sterilized in an autoclave for fifteen to twenty minutes.

Different kinds of tests were carried out to determine the efficiency of the cannula in preventing contamination of syringe contents. Included were in vitro tests in which bacterial culture media were employed as indicators of contamination of syringe contents following removal of an artificially contaminated needle, tests in which laboratory animals served as the indicator, tests in which the chorio-allantoic membrane of developing duck eggs served as the indicator, and tests carried out on human beings.

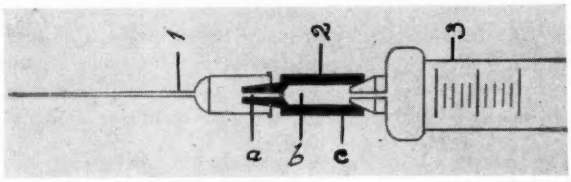



Fig. 1.—*1*, Needle. *2*, "Cannula." *3*, Syringe. *a*, Narrow channel at the distal end, directly joining the needle. *b*, Wider channel joining the syringe. *c*, Brass tube.

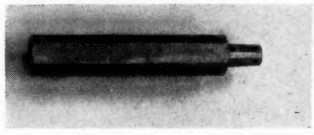


Fig. 2.—The cannula.

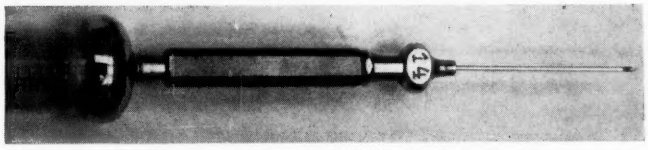


Fig. 3.—The cannula fitted to needle and syringe.

*a. In Vitro Tests.*—The syringes were filled with 0.5 to 5 ml. of sterile nutrient broth, or with a heat-killed twenty-four-hour broth culture of staphylococci, or with physiologic saline solution, and were immediately provided with sterile needles attached to the cannulas, after which the air was removed from the syringe and needle. Artificial contamination was effected by immersing the distal one-third of the needle in a broth culture of the test bacterium for about three seconds. The test bacteria employed were *Serratia marcescens* and *Vibrio El Tor*, the latter a highly motile organism. In imitation of an injection in a mass immunization campaign, 0.5 to 5 ml. of the liquid in the syringe was then injected subcutaneously into a monkey, except that in Experiment 2, the injection was made intravenously into a rabbit and in Experiment 11 the liquid was injected intramuscularly. In all, eleven experiments were carried out, each involving a number of individual tests (see

Table I); with the exception of certain experiments (Experiments 2, 7, 8, and 11), the injection was followed by a deeper insertion of the needle into the tissues, amounting to about 0.25 cm. or more. Following the deeper insertion the syringe was loosened from the needle-cannula assembly, and the contents (±0.5 ml.) of the syringe was inoculated onto nutrient agar. Then, while the needle was still in the tissue, the liquid in the needle was sucked away with another syringe and this also was inoculated onto nutrient agar. The agar plate cultures were incubated at room temperature and were read at the end of 24 and 48 hours. In the case of Experiment 2 in which the injection was made into an ear vein of the rabbit, firm pressure was promptly applied with thumb and finger where the needle point was situated in order to increase the tissue pressure around the needle opening. In Experiment 7 the needle was not pressed deeper into the tissue following the subcutaneous injection; in Experiment 8 the needle, while still attached to the cannula-syringe assembly, was pulled out immediately after the injection and then the syringe was immediately detached from the needle-cannula assembly; in Experiment 9, 0.1 or 0.2 ml. of the liquid was injected intradermally; in Experiment 10 the syringe was filled with 6 ml. of liquid and injected in fractions of 1 ml. subcutaneously, each injection being followed by deeper insertion of the needle into the tissues, and following each of which the used syringe was immediately provided with a fresh cannula-needle assembly and then the needle was artificially contaminated with the test bacterium. Experiments 3 and 11 were alike, except that the amount of liquid injected differed. In all of the experiments except one (Experiment 4) the test bacterium was *S. marcescens*, the test organism in this exception being *Vibrio El Tor*. Sliding or drawing back of the plunger after the artificial contamination of the needle should not be permitted since this action would allow the infected material in the needle to be sucked back into the syringe. This can be avoided by holding the syringe during the operation with its forward end slanting downward.

*b. Infection Tests on Animals.*—The reliability of the cannula in preventing contamination of syringe contents was also tested out in different kinds of animals with infectious agents to which they were known to be highly susceptible, including a strain of Streptococcus (Aronson), obtained through the courtesy of Professor R. Gispen, and a strain of *Diplococcus pneumoniae* (Type III), both of which were used with mice as indicator animals, and a strain of *Mycobacterium tuberculosis* (human type), which was used with guinea pigs as indicator animals. In addition, vaccinia virus was employed as a test organism, with the chorio-allantoic membrane of developing duck eggs serving as the indicator of whether or not the fluid contents of the syringe had become contaminated with the virus (Table II).

In carrying out these tests the procedure was essentially the same as described under "in vitro tests" except that here the syringe contents was tested for sterility by injection into appropriate animals. After the end of the needle was contaminated with either the streptococcus or the pneumococcus, 1.5 ml. of the fluid contents of the syringe (±1.75 ml. of sterile nutrient broth or physiologic saline solution) was injected deep subcutaneously into a normal monkey, with the subsequent deeper insertion previously described. The syringe was loosened from the needle-cannula assembly and the syringe fitted with a new needle. The remaining contents of the syringe (0.25 ml.) was then injected intraperitoneally into a white mouse. The inoculated mice were kept under observation for ten days and those that died were examined for infection with the test bacterium. Control tests were carried out as described previously, except that the cannula was not employed.

In the tests carried out with the tubercle bacillus, the organism was cultivated on Löwenstein-Jensen medium and a suspension of organism in broth was prepared. The procedure from here on was the same as that described previously except that the remaining contents of the syringe (0.5 ml.) was injected deep intramuscularly into the thigh of a guinea pig, after which the animal was kept under observation for three months. Control tests without the use of the cannula were also carried out here.

In the tests carried out with vaccinia virus, 20 doses of dried vaccine were ground finely in a mortar and suspended in 2 ml. of sterile broth. The individual tests were carried out as in the above tests, except that the residual fluid in the syringe (0.2 ml.) was inocu-

lated onto the chorio-allantoic membrane of a 2-day-old duck egg embryo. During the following week pock lesions were looked for on the membrane, and smears were stained for Paschen bodies. Control tests were carried out omitting the use of the cannula. For further particulars regarding the above tests see Table II.

TABLE II. TEST IN HUMAN BEINGS AND IN ANIMALS, WITH OR WITHOUT "CANNULA" BETWEEN NEEDLE AND SYRINGE

| EXPERIMENT NO. | TEST BACTERIUM | ROUTE AND ANIMAL | WITH CANNULA | | WITHOUT CANNULA | |
|---|---|---|---|---|---|---|
| | | | NUMBER TESTS | NUMBER POSITIVES (%) | NUMBER TESTS | NUMBER POSITIVES (%) |
| 1a | Streptococcus | Intraperitoneal; Mice | 60 | 0 | 34 | 88 |
| 1b | *D. pneumoniae* | Subcutaneous or intraperitoneal; Mice | 20 | 0 | 20 | 55 |
| 2 | *Myco. tuberculosis* | Deep subcutaneous; Guinea pigs | 10 | 0 | 10 | 10 |
| 3 | Vaccinia virus | Inoculation on chorio-allontoic Membrane of Eggs | 20 | 0 | 10 | 70 |
| 4 | Avirulent *Past. pestis* (Tjiwidei) | Inoculation on agar; Human beings | 200* | 0 | 160 | 31 |
| | | Total | 310 | 0 | 234 | 51 |

*On fifty human beings.

*c. Tests on Human Beings.*—Tests were carried out on human beings in which use was made of an avirulent strain of *Pasteurella pestis* (Tjiwidei strain) as the test organism. This strain was obtained through the courtesy of Professor Moh. Djoehana Wiradikarta of the Pasteur Institute, at Bandung, Indonesia, and is a strain employed there in the preparation of living plaque vaccine. It was employed as a forty-eight-hour broth culture for contamination of the needle. In other respects the procedure was like that already described in carrying out the cannula efficiency tests. After the artificial contamination of the end of the needle 2 to 3 ml. of sterile physiologic saline solution in the syringe was injected into a human being, and contents of the syringe and the needle were checked for the presence of organisms by inoculation onto nutrient agar. Control tests without the use of a cannula were also carried out. For further particulars of these tests see Table II.

It was also important to see to what extent this new method of injection was reliable when carried out by medical personnel. The personnel selected were an experienced chief hospital attendant, a medical student and an assistant laboratory worker.

## RESULTS

The results of the tests described above are given in Tables I and II. Little need be added here regarding these results. They show clearly that the use of the special cannula of at least 0.15 ml. capacity does provide an added margin of safety and they suggest that with the proper use of such a cannula complete safety is assured in the use of a single syringe for multiple subcutaneous or intracutaneous injections of 0.1 ml. to a maximum of 5 ml. of liquid. There is apparently no danger of a contamination of syringe contents because of the active movement of microorganism from the needle into the syringe or because of muscle contractions during the injection. The incidence of contamination in tests in which no cannula was used was high.

## DISCUSSION

It should be emphasized that in the employment of such a cannula both syringe and needle should fit well onto the cannula. Leaky cannulas or

syringes should not be employed. The prevention of the backflow of contaminated liquid into a syringe is not only based on the extension of the distance between syringe and needle by means of the cannula, but is also based on holding the cannula-needle assembly (after deeper insertion of ±0.25 cm. or more deeper into the tissue) in the tissue during the loosening of the syringe from the needle-cannula assembly following the injection. In this way the tissue functions more or less as a valve to the needle opening. The details described under Methods regarding the application of the cannula should be carefully observed.

SUMMARY

A cannula is described which, when inserted between the syringe and needle, completely eliminates the risk of contamination of syringe contents when a single syringe is used for the injection of a number of persons in mass immunization campaign. Extensive experimental observations are presented in support of this claim and the requirements for the successful use of the cannula are discussed.

I would like to express my appreciation for the advice and assistance in preparing this paper given by Dr. E. W. Schultz, Visiting Professor of Pathology, and Dr. Thomas N. Burbridge.

REFERENCES

1. Evans, R. J., and Spooner, E. T. C.: Possible Mode of Transfer of Injection by Syringes Used for Mass Inoculation, Brit. M. J. **2**: 185-188, 1950.
2. Findlay, G. M., and MacCallum, F. O.: Note on Acute Hepatitis and Yellow Fever Immunization, Tr. Roy. Soc. Trop. Med. & Hyg. **31**: 297-380, 1937.
3. Fleming, A., and Ogilvie, A. C.: Syringes, Needles, and Mass Inoculation Technique, Brit. M. J. **1**: 543-546, 1951.
4. Gispen, R.: A One-Way Valve for Multiple Injections From One Syringe, Lancet **2**: 171-172, 1952.
5. Gan K. H., and Tjioe Swie Bie: The Danger of Contamination of Syringe Contents in the Use of One Syringe for Multiple Injections, J. Indonesian M. A. **3**: 441-450, 1953.
6. Hughes, R.: Post-penicillin Jaundice, Brit. M. J. **2**: 685-688, 1946.

# CORRECTION OF PAPER-STRIP SERUM ELECTROPHORESIS DIAGRAMS

A. L. Latner, M.Sc., M.D., D.I.C., F.R.I.C.
Newcastle Upon Tyne, England

TECHNIQUES of paper-strip electrophoresis of serum proteins have been developed at a number of different centers. Individual investigators have obtained considerable practice in their own particular methods, especially in regard to their protein stain and their scanning procedure. These workers are naturally rather loath to discard such valuable experience. Much argument has therefore arisen in relation to the special qualities of certain stains. A number have been criticized, sometimes incorrectly, on the ground that they do not give straight-line relationships between staining intensity and protein concentration.[1]

In order to overcome these arguments, the correction principle described independently by Crook and his associates[1] has been used by me for a considerable time. It has been incorporated in a scanning device elaborated at this school.[2] It can be applied, in the form of a Perspex triangle, to the correction of any electrophoresis curve.

In the first place, it is necessary to determine the relationship between protein staining and protein concentration. This can be done quite simply using the "paper photo-wedge" technique I have described elsewhere.[2, 3] This technique is preferable to that described by Crook and his associates[1] because of the greater tendency of that method to produce air bubbles in the paper. A curve can be obtained by plotting serum-protein concentration as abscissa against the logarithm of the ratio of the intensity of light transmitted through unimpregnated paper to that through paper impregnated with protein at a given concentration. If so desired, reflected light can also be used. The scale of the ordinate must be identical with that obtained by the scanning device.

The resulting curve will usually be found to approximate closely to an oblique straight line at the lower protein concentrations and then to flatten out and become curvilinear. By means of a ruler the straight-line portion is continued upward as an oblique dotted line. Alternatively, if the original graph were entirely curvilinear, the dotted line would be tangential to the early portion of the curve. A perpendicular is dropped from the dotted line so as to include all of the experimentally determined curve (Fig. 1). The resulting right-angled triangle and its contained curve lying partly along and partly just under the hypotenuse can then be reproduced in transparent Perspex. The curve, is, of course, engraved on the triangle with a sharp-pointed instrument, preferably on the undersurface to avoid parallax.

From the Section of Chemical Pathology, Department of Pathology, King's College and Royal Victoria Infirmary, Newcastle upon Tyne, England.
Received for publication July 13, 1954.

A serum paper-strip electrophoresis diagram can now be corrected by sliding the side of the Perspex triangle representing the original abscissa along the base line of the electrophoresis diagram. Wherever the curve engraved on the Perspex triangle cuts the electrophoresis tracing, the point is replotted on the hypotenuse vertically above the point of intersection. When a sufficient number of points have been obtained, the correct electrophoresis diagram can be drawn. It is a great help if a series of vertical lines, 2 mm. apart, are also engraved on the undersurface of the triangle.

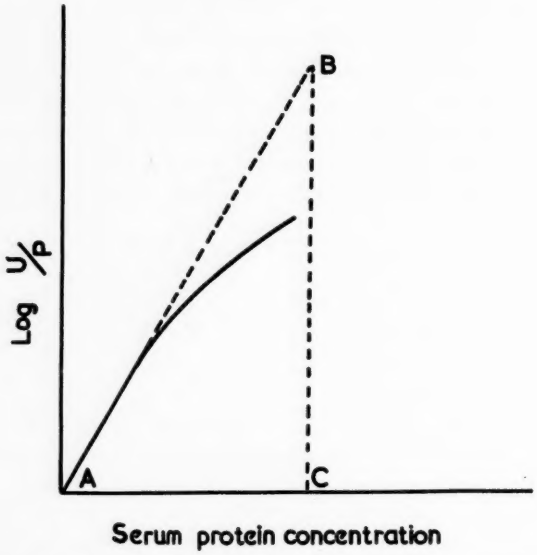



Fig. 1.—Hypothetical curve obtained by plotting protein concentration against log U/P. The right-angled triangle ABC is subsequently reproduced in Perspex. U = Intensity of light transmitted through unimpregnated paper. P = Intensity of light transmitted through protein-impregnated paper.

Protein concentrations can now be obtained from the areas under the peaks. With certain stains having different affinities for albumin and globulin an additional correction factor must be employed.

There are obviously applications to a number of other types of investigation requiring the conversion of a curve to an ideal straight line.

Experience has shown that the correction device is not necessary when using my staining technique with Naphthalene Black 12 B 200[2, 3] and the scanning instruments described elsewhere.[2, 4]

REFERENCES

1. Crook, E. M., Harris, H., Hassan, F., and Warren, F. L.: Continuous Direct Photometry of Dyed Materials in Filter Paper With Special Reference to the Estimation of Proteins Separated by Electrophoresis, Biochem. J. **56:** 434-444, 1954.
2. Latner A. L., Molyneux, L., and Rose, J. D.: A Semiautomatic Recording Densitometer for Use After Paper Strip Electrophoresis, J. Lab. & Clin. Med. **43:** 157-164, 1954.
3. Latner, A. L.: A Quantitative Study in Relation to Electrophoresis of the Staining of Serum Proteins on Filter Paper With Naphthalene Black, Biochem. J. **52:** xxix, 1952 (Abstract).
4. Latner, A. L.: Conversion of the Spekker Absorptiometer to a Semi-automatic Scanning Device for Quantitative Electrophoresis of Serum Proteins on Filter Paper, Biochem. J. **52:** xxxii, 1952 (Abstract).

# THE MANOMETRIC ANALYSIS OF RESPIRATORY GASES IN BLOOD CONTAINING VOLATILE ANESTHETIC AGENTS

## I. A Comparison of Macromethods

Duncan A. Holaday, M.D., and Mariagnes Verosky, B.A.
New York, N. Y.

THE Van Slyke-Neill[1] manometric method is not applicable to the analysis of respiratory gases of blood samples which contain volatile anesthetic agents because the latter, due to their high water solubilities, are redistributed between the gaseous and liquid phases within the reaction chamber following the introduction of absorbing solutions. Numerous attempts have been made to eliminate or to compensate for the artefact caused by the redistribution of extraneous gases. The present study was undertaken to explore the reliability of two methods.

Goldstein and associates[2] have devised a method suitable for the combined determination of oxygen and carbon dioxide content of blood which contains low concentrations of ethyl ether. Their method is based on the assumption that redistribution of ether continues at a constant rate for approximately two minutes following the introduction of sodium hydroxide. The extraction period following the reading of $P_1$ is divided into two equal one-minute periods, during the first of which all of the carbon dioxide is absorbed. Subtraction of the pressure change noted during the second period from that of the first period corrects for the redistribution of the ether. Because the water solubility of ether exceeds that of the other commonly used anesthetic agents, it was felt that this method should be applicable to analysis of bloods from all anesthetized subjects. Accordingly, the Goldstein method was compared with the method of Orcutt and Waters,[3] the latter being the previously preferred method in this laboratory for analysis of $O_2$ and $CO_2$ in blood samples containing nitrous oxide or cyclopropane.

### METHODS

Two hundred and sixty-four determinations representing duplicate analyses of 66 blood samples by two methods constitute the basis for this report. The Van Slyke-Neill method was used as the standard of reference for estimating the accuracy of the Goldstein method and the Orcutt and Waters method on bloods free from volatile agents. The Goldstein and the Orcutt and Waters methods were compared directly on blood samples obtained from patients during surgical anesthesia induced with nitrous oxide, cyclopropane, or ethylene. Ten-milliliter blood samples were drawn into sealed syringes and were refrigerated. The bloods were mixed for three minutes immediately before each determination.

From the Department of Anesthesiology, Columbia University, College of Physicians and Surgeons, and the Anesthesiology Service, The Presbyterian Hospital, New York 32, N. Y.

Aided by a medical research grant from the National Tuberculosis Association, through its Medical Section, the American Trudeau Society.

Received for publication July 14, 1954.

The several methods were employed essentially unmodified for the determination of oxygen and carbon dioxide content. The Goldstein method was adapted for the estimation of content of agent by carefully expelling the bubble of gas after $P_3$ is read; the liquid level is then returned to the 2 ml. mark and $P_4$ is read. The conversion factors for cyclopropane, nitrous oxide, and ethylene, prepared by Orcutt and Waters for their method,[3] were used to calculate the gas volumes according to the formula: $(P_3 - P_4) \times F =$ content of agent, in which "F" represents the appropriate conversion factor.

To determine the accuracy of the estimations of carbon dioxide and oxygen contents of blood containing volatile anesthetic agents, bloods free of agent were analyzed in the usual manner, then reanalyzed in the presence of cyclopropane. The following method was devised to obtain the mixture without altering the $O_2$ or $CO_2$ contents of the sample: the gas-releasing solution (ferricyanide, saponin, and lactic acid) was degassed in the Van Slyke chamber as usual, and partially returned to the cup. The pressure in the chamber was then reduced to a manometer reading similar to the usual $P_3$ reading. The leveling stopcock was closed and the leveling bulb returned to the upper position. A rubber-clad glass tube connected by a heavy-walled rubber tubing to a tonometer containing pure cyclopropane was then pressed snugly into the bottom of the cup (after thorough rinsing of the connecting tube with the cyclopropane). The chamber was then opened cautiously to the cup and the pressure was allowed to rise about 15 mm. Hg. A mercury seal was obtained, and the leveling stopcock was opened to release the vacuum in the chamber. The usual method was then continued, starting with the introduction of the blood sample and the appropriate volume of gas-releasing solution.

## RESULTS

The Goldstein method produced results indistinguishable from those obtained by the Van Slyke-Neill method with blood samples drawn from subjects breathing room air (Table I). By the method of Orcutt and Waters, a similar group of blood samples appeared to contain 0.4 per cent by volume of $CO_2$ more, and 0.4 per cent by volume of $O_2$ less than that estimated by the Van Slyke-Neill method. The average of the estimates obtained by the Orcutt

TABLE I. DIFFERENCES BETWEEN METHODS

| METHOD | AGENT | n | $CO_2$ (VOL. %) | $O_2$ (VOL. %) | AGENT (VOL. %) |
|---|---|---|---|---|---|
| V.S.-N minus G. | None | 20 | +0.01 ± 0.11 | +0.14 ± 0.09 | |
| V.S.-N. minus O. and W. | None | 22 | −0.41* ± 0.08 | +0.44* ± 0.06 | |
| G. minus O. and W. | $N_2O$ | 10 | −0.19 ± 0.12 | +0.06 ± 0.12 | 0.00 ± 0.06 |
| G. minus O. and W. | Cyclopropane | 8 | −0.25* ± 0.10 | +0.18 ± 0.14 | +0.01 ± 0.08 |
| G. minus O. and W. | Ethylene | 6 | −0.28* ± 0.05 | +0.12 ± 0.05 | +0.15* ± 0.04 |
| G. minus O. and W. | All agents | 24 | −0.23* ± 0.06 | +0.11 ± 0.07 | +0.03 ± 0.03 |

Figures represent the means of the differences between the contents estimated for each blood sample by the indicated methods, and the standard error of each mean difference. "n" is the number of samples included in each comparison; each sample was analyzed in duplicate by two methods. Asterisks indicate significant differences, i.e., the probability that the difference could have occurred by chance alone is less than 0.02 as determined by the "t" test.

and Waters method and by the Goldstein method of the $O_2$ and $CO_2$ contents of blood samples containing between 2.4 and 32.7 per cent by volume of agent showed similar differences, but of a smaller magnitude. The estimates of the content of agents by the two methods agreed well. The presence of anesthetic agent did not introduce a bias into the estimations of $CO_2$ or $O_2$ by either method (Table II).

TABLE II

| METHOD | $CO_2$ (VOL. %) | $O_2$ (VOL. %) | AGENT (VOL. %) |
|---|---|---|---|
| Goldstein | 47.5 | 13.2 | None |
| | 47.4 | 13.4 | None |
| | (Prior to each of the following determinations, the indicated amount of cyclopropane was added to an aliquot of the sample within the Van Slyke chamber.) | | |
| Goldstein | 47.6 | 13.6 | 11.3 |
| | 47.5 | 13.5 | 11.3 |
| Orcutt and Waters | 47.8 | 13.6 | 11.3 |
| | 48.1 | 13.4 | 12.8 |

Consecutive analyses of a single sample of blood in the absence and in the presence of cyclopropane. Refer to text for details of method.

The sensitivities of the three methods are similar as measured by the estimated standard deviation of individual determinations based on differences between duplicate determinations (Table III).

TABLE III. COMPARISON OF THE VARIANCES OF THE METHODS

| | WITHOUT AGENT | | | WITH AGENT | | | |
|---|---|---|---|---|---|---|---|
| METHOD | n | $CO_2$ (VOL. %) | $O_2$ (VOL. %) | n | $CO_2$ (VOL. %) | $O_2$ (VOL. %) | AGENT |
| Van Slyke-Neill | 22 | 0.22 | 0.30 | | | | |
| Goldstein | 12 | 0.23 | 0.20 | 24 | 0.27 | 0.18 | 0.20 |
| Orcutt and Waters | 12 | 0.22 | 0.24 | 24 | 0.23 | 0.27 | 0.24 |

The figures represent the standard deviations of each method based on the differences between duplicate determinations (s.d. $= \sqrt{\frac{(\text{diffs})^2}{2n}}$, where n = number of samples). There are no significant differences between the sensitivities of the several methods with the possible exception of the values obtained for the determination of $O_2$ in the presence of agent by the Goldstein and the Orcutt and Waters methods. Application of the "F" test in this instance indicates that chance alone might account for the difference observed more than once but less than five times out of 100 similar comparisons.

Goldstein and associates[2] cite evidence that oxygen is consumed in the presence of alkaline ferricyanide, caprylic alcohol, and saponin, and present data supporting the view that their method corrects for this source of error. Additional evidence concerning this was obtained during the present study. In the absence of volatile anesthetic agents the average loss of pressure between $P_2'$ and $P_2''$ was 0.5 mm. Hg, with a standard deviation of ± 0.15 mm. Hg, in twenty consecutive determinations on twenty blood samples. The average difference between $P_2'$ and $P_2''$ in forty analyses of the plasma obtained from the same twenty blood samples was 0.015 mm. Hg ± 0.08. The same reagents were used for both determinations.

## DISCUSSION

The results of the present study indicate that estimations of $O_2$ and $CO_2$ content of blood from subjects breathing air obtained by the Goldstein method and the Van Slyke-Neill method agree well, whereas the method of Orcott and Waters consistently yields higher $CO_2$ contents and lower $O_2$ contents.

The differences noted can be accounted for in terms of consumption of oxygen during the period of extraction in alkaline ferricyanide. In the Van

Slyke-Neill method $P_2$ is read very shortly following introduction of sodium hydroxide, thus preventing significant consumption of oxygen. In the Goldstein method the extent of consumption of oxygen occurring during the first period of extraction following introduction of sodium hydroxide is obscured by simultaneous absorption of carbon dioxide. This loss is measured during a second period of extraction before the oxygen absorber is added, and correction is made. In the method of Orcutt and Waters extraction is continued for three minutes following introduction of sodium hydroxide and before $P_2$ is read. Any oxygen consumed during this period will appear in the calculations as $CO_2$ absorbed. In the Goldstein method the reduction of pressure in the chamber observed during the second period of extraction after addition of sodium hydroxide is of an appropriate order of magnitude to account for the differences noted between the two methods.

## CONCLUSIONS AND SUMMARY

The method of Goldstein and associates for the combined determination of oxygen and carbon dioxide in blood containing ether has been found to be suitable for use on bloods containing any of the volatile anesthetic agents. The method is easy to learn and is reliable. The authors have found it expedient to use the Goldstein method on all bloods analyzed regardless of whether or not anesthetic agents are present.

The method of Orcutt and Waters probably contains a systematic error due to the consumption of oxygen in the presence of alkaline ferricyanide, caprylic alcohol, and saponin, which results in erroneously high estimations of carbon dioxide content, and erroneously low estimations of oxygen.

## REFERENCES

1. Van Slyke, D. D., and Neill, J. M.: The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement. I., J. Biol. Chem. **61:** 523-573, 1924.
2. Goldstein, F., Gibbon, J. H., Jr., Allbritten, F. F., Jr., and Stayman, J. W., Jr.: The Combined Manometric Determination of Oxygen and Carbon Dioxide in Blood in the Presence of Low Concentrations of Ethyl Ether, J. Biol. Chem. **182:** 815-820, 1950.
3. Orcutt, F. S., and Waters, R. M.: A Method for the Determination of Cyclopropane, Ethylene, and Nitrous Oxide in Blood With the Van Slyke-Neill Manometric Apparatus, J. Biol. Chem. **117:** 509-515, 1937.

# IMPROVED RESISTANCE WIRE STRAIN-GAUGE MANOMETERS ADAPTABLE FOR BIOLOGIC MEASUREMENTS

EARL H. WOOD, M.D., PH.D., AND WILLIAM SUTTERER, B.S.
ROCHESTER, MINN.

SINCE its adaptation for measurement of arterial blood pressure in 1946,[1-3] various types of Statham strain-gauge manometers have been used successfully in many laboratories for measurement of biologic pressures.[4] As pointed out in early communications,[3] the chief disadvantage of this gauge when used for recording of arterial pressure has been its relatively low natural frequency.[3, 4]

## METHODS AND RESULTS

It is the purpose of this paper to describe two new unbonded strain-gauge manometers[5, 6] with sensitivity and dynamic response characteristics that are considerably superior to those of the standard (±780 mm. Hg) Statham gauge.*

The improved gauges, Type 4-310 and P23D, are manufactured by the Consolidated Engineering Corporation† and Statham Laboratories, respectively.‡ The internal features of the Type 4-310 gauge are shown in Fig. 1. The

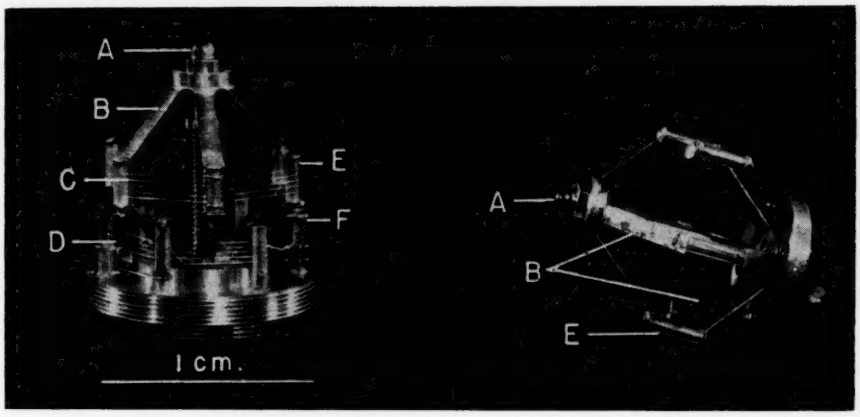



Fig. 1.—Internal features of Consolidated 4-310 star-type pressure transducer; complete assembly on left, star spring assembly on right. The two active arms (*C*) of the four-arm Wheatstone bridge are wound around the vertical pins (*E*) joining the two star-shaped springs (*B*). Pressure on the diaphragm (not shown), which rests against the ball (*A*) atop the upper spring, depresses the entire structure. The resultant spreading of the pins stretches the strain-sensitive wires of the two active arms (*C*) to cause a bridge unbalance and output proportional to the pressure. The two inactive bridge arms (*D*) wound around the base pins (*F*) are unaffected.

Due to the geometry of the spring suspension system and arrangement of the windings, axial and transverse accelerations have little effect on bridge output, for they are self-canceling. Temperature compensation for zero drift over a temperature range of −54° to +110° C. is accomplished by a resistance connected in series with one of the inactive bridge arms.

From the Section of Physiology, Mayo Clinic and Mayo Foundation, Rochester, Minn. The Mayo Foundation is a part of the Graduate School of the University of Minnesota.

Received for publication July 26, 1954.

*The specially adapted model P6-15G-250 gauge used was manufactured by the Statham Laboratories, Los Angeles, Calif.

†Located at 300 North Sierra Madre Villa, Pasadena 8, Calif.

‡Located at 12401 West Olympic Boulevard, Los Angeles 64, Calif.

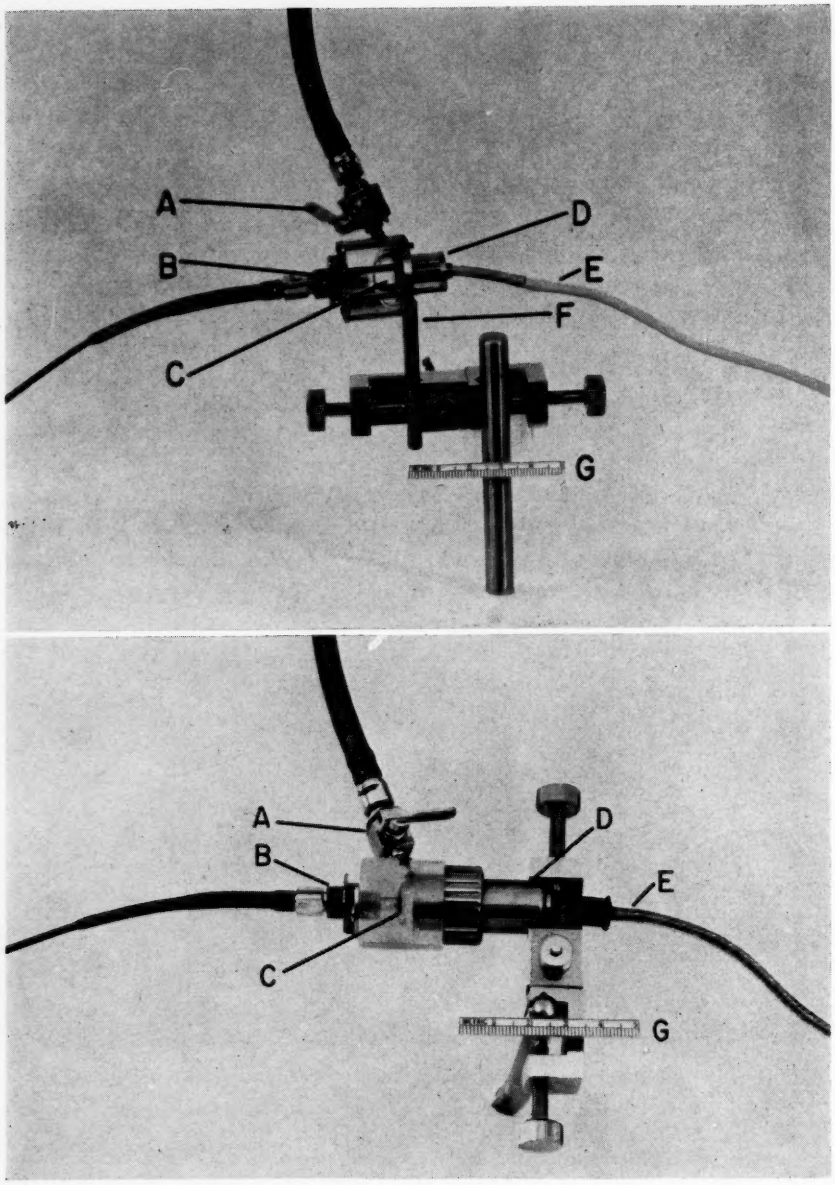



Fig. 2.—Strain-gauge manometers with specially designed Lucite adapters for biologic applications. Top: Consolidated Type 4-310 gauge. Bottom: Statham Type P23D gauge. *A*, Stopcock connecting pressurized wash bottle to pressure chamber of manometer. The wash bottle contains sterile 0.9 per cent solution of sodium chloride with 20 mg. of sodium heparin per liter. The pressure chamber of the manometer, hydraulic connections, and catheter or needle are washed free of air bubbles and kept free from formation of blood clots by frequent flushing of the hydraulic system with this solution. *B*, Male Luer-Lok adapter connected to a No. 6 Cournand catheter for recording intracardiac pressures.[8] The adapter is provided with external threads for making rigid (low-compliance) connections to special low-compliance stopcocks. *C*, Pressure chamber covering membrane of manometer and filled with sterile solution of sodium chloride. This Lucite chamber is domed and polished for ease of visualization and removal of minute air bubbles. *D*, Strain-gauge manometer proper. *E*, Four-wire electric cable to gauge. *F*, Steel mounting rod. *G*, Centimeter scale.

internal construction of the P23D gauge is similar but much smaller than the standard Statham gauge.[4] Lucite adapters were constructed for both of these gauges for use in biologic studies. These adapters* are equipped with a Luer-Lok hypodermic fitting for connection of catheters or needles and a stopcock for flushing the hydraulic system free of air bubbles and blood clots (Fig. 2).

A comparison of the physical characteristics of the Consolidated and Statham gauges is given in Table I. The sensitivities of the Consolidated 4-310 and the new P23D Statham gauges are more than twice that of the old Type P6 (±780 mm. Hg) Statham gauge in spite of the fact that the elasticity coefficients ($\Delta P/\Delta V$) have been increased by factors of 2 and more than 6, respectively, and hence the resonant frequencies by approximately 1.4 and 2.5, respectively, for the 4-310 and P23D gauges when identical hydraulic connections are used.

TABLE I. COMPARISON OF PHYSICAL CHARACTERISTICS OF STATHAM AND CONSOLIDATED STRAIN GAUGES

| CHARACTERISTIC | TYPE OF GAUGE | | |
|---|---|---|---|
| | STATHAM P6-15G-250 | CONSOLIDATED 4-310 | STATHAM P23D |
| Pressure range, mm. Hg | ±780 | −250 to +780 | ±780 |
| Displaced volume ($\Delta V$), cu. mm./100 mm. Hg | 0.364 | 0.182 | 0.059 |
| Volume elasticity coefficient ($\Delta P/\Delta V$), $10^8$ dynes/cm.$^5$ | 3.65 | 7.32 | 24.1 |
| Resonant frequency with 20-gauge, 5-cm. needle (0.056 cm. internal diameter), cycles per second | 70 | 100 | 180 |
| Bridge resistance, ohms | 240 | 350 | 390 |
| Input voltage, volts D.C., max. | 10 | 10 | 10 |
| Current, milliamperes | 41 | 28.5 | 25.5 |
| Output closed circuit, microamperes/100 mm. mercury/10 volts | 10 | 15 | 15 |
| Open circuit voltage, millivolts/100 mm. mercury/10 volts | 2.3 | 5.1 | 5.7 |
| Calibration, full scale | linear | linear | linear |
| Hysteresis, per cent of $\Delta P$ | <1 | <1 | <1 |

For these comparative studies the outputs of the three gauges were recorded photographically by a suitable galvanometer† without electronic amplification. The dynamic response characteristics of the gauges were determined using an electromagnetic pressure oscillator.[8-10] When the Types P6, 4-310, and P23D

*Designed and constructed by the Section of Engineering, Mayo Clinic, Rochester, Minn.

†A Heiland G-150 galvanometer was used. It had the following characteristics: natural frequency, 150 cycles per second; optimal damping resistance, 400 ohms; sensitivity, 0.4 microampere per millimeter at 1 meter. It was manufactured by the Heiland Research Corporation, Denver, Colo.

gauges were connected directly to a 20-gauge, 5-cm. hypodermic needle, their resonant frequencies were 70, 100, and 180 cycles per second and the damping coefficients were 0.13, 0.10, and 0.08, respectively (Fig. 3). When these gauges were connected to a plastic, arterial-type catheter 80 cm. in length and 0.5 mm. in internal diameter, these frequencies were reduced to 12, 20, and 40 cycles per second and the damping coefficients increased to 0.39, 0.32, and 0.20, respectively (Fig. 3).

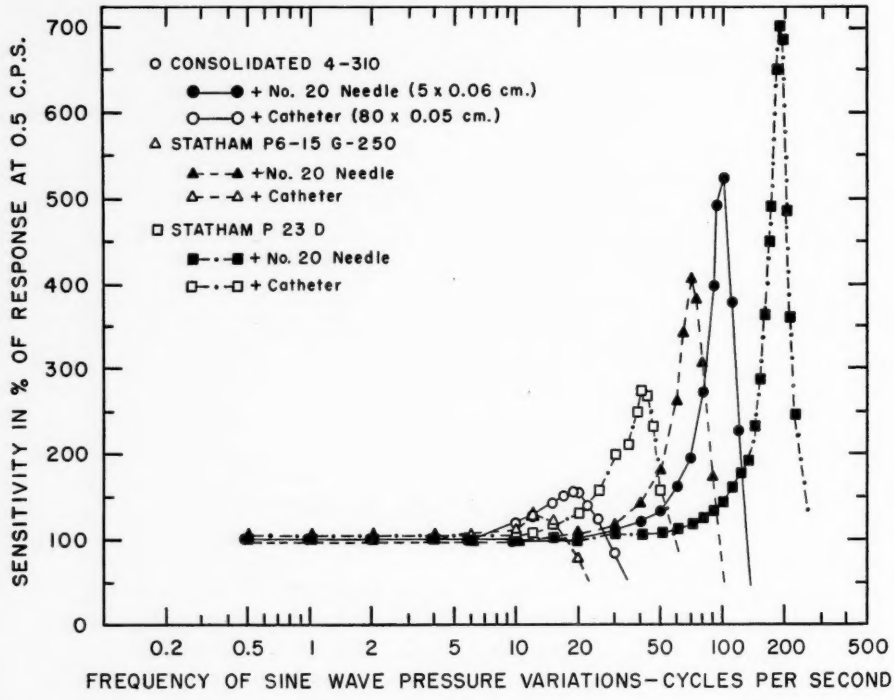



Fig. 3.—Comparison of dynamic response of Statham and Consolidated ±780 mm. Hg strain gauges when used with identical external hydraulic connections consisting of a 20-gauge needle or an 80-cm. arterial type catheter.

For recording of central arterial pulse contours in man, the output of the Consolidated gauge is recorded by a special galvanometer* with a natural frequency of 21 cycles per second. Proper electromagnetic damping of this galvanometer provides an optimally damped arterial catheter-manometer system with a flat response (±5 per cent) up to 17 cycles per second. Immediately consecutive recordings of aortic pressure pulses in man by a high frequency capacitance manometer† and Consolidated manometer-catheter systems revealed no practically significant differences in the pressures or contour of the aortic pulses recorded by the two manometer-catheter systems.[12, 13] Utilizing the

*This is a Heiland Type E galvanometer with added length and mass on the suspension to give the following characteristics: natural frequency, 21.5 cycles per second; optimal damping resistance, 1,600 ohms; sensitivity, 0.06 microampere per millimeter at 1 meter.

†This is a Lilly type capacitance manometer manufactured by the Technitrol Company, Philadelphia, Pa. The volume elasticity coefficient[11] $= 83 \times \frac{10^8}{cm.^5}$.

P23D gauge and a combination of mechanical and electric damping, arterial-catheter manometer systems can be assembled with a flat response to 40 cycles per second.

## COMMENT

Unbonded strain-gauge manometers with direct galvanometric recording and without electronic amplification are the simplest, most foolproof electric pressure transducers now available. For use in this simple electric manometer system, the strain gauge must be of sufficient sensitivity to produce adequate deflections of the recording galvanometer. Since sensitivity varies inversely with the stiffness of the manometer, the limiting factors in determining the natural frequency of manometers suitable for measuring arterial pressure are the sensitivities of the strain gauge and the recording galvanometer. The high sensitivities of the Consolidated 4-310 and Statham P23D gauges make possible, therefore, the use of considerably stiffer and hence higher frequency strain-gauge systems for this purpose. This increase in frequency will be of particular value in the recording of pressures via long narrow-bore catheters, as is often necessary in hemodynamic studies in man.

The sensitivity of the P23D gauge when utilized with high performance galvanometers and without electronic amplification is several times greater than that required for arterial pressure pulse recording in man. It should be possible therefore to construct similar gauges whose resonant frequencies will compare favorably with available high frequency electronically amplified capacitance manometers.[11, 14] The elimination of the necessity for the expense and complications of vacuum-tube amplification made possible by these improved strain gauges is a matter of considerable practical importance.

## SUMMARY

Comparative studies of the physical characteristics of old type Statham and new type Consolidated and Statham unbonded strain gauges are reported. The new type Statham P23D* strain gauge was found to be superior, possessing approximately twice the sensitivity and 2.6 times the resonant frequency of the P6 type Statham (±780/mm. Hg) gauge when identical hydraulic connections were used.

The high sensitivities of the Consolidated Type 4-310 and Statham P23D gauges permit use of considerably higher frequency strain-gauge manometers for biologic measurements than has been possible heretofore. High resonant frequency coupled with greater simplicity and superior stability characteristics render these resistance wire manometers equivalent or superior for biologic applications to other types of commercially available electric transducers.

## REFERENCES

1. Lambert, E. H., and Wood, E. H.: Direct Determination of Man's Blood Pressure on the Human Centrifuge During Positive Acceleration, Fed. Proc. 5: 59, 1946.

*A commercial version of the P23D gauge with an adapter for biologic use is available from the Statham Company.

2. Braunstein, J. R., Brosene, W. G., Jr., Ablendi, Frank, Green, R. S., Strauss, Victor, Hauenstein, Virgil, and Kersten, H. J.: A New Method of Recording of Arterial Blood Pressure, Science **105**: 267, 1947.
3. Lambert, E. H., and Wood, E. H.: Use of Resistance Wire, Strain Gauge Manometer to Measure Intra-arterial Pressure, Proc. Soc. Exper. Biol. & Med. **64**: 186-190, 1947.
4. Lambert, E. H.: Strain Gauges: Resistance Wire. In Glasser, Otto: Medical Physics, Chicago, 1950, Year Book Publishers, Inc., vol. 2, pp. 1090-1098.
5. Consolidated Engineering Corporation: Type 4-310 Star-type Pressure Pickup Bulletin, CEC 1534, Pasadena 15, California.
6. Statham Scientific Instruments: The Model P23D Physiological Pressure Transducer, May, 1954, Los Angeles 64, California.
7. Wood, E. H.: Special Technics of Value in the Cardiac Catheterization Laboratory, Proc. Staff Meet., Mayo Clin. **28**: 58-64, 1953.
8. Wood, E. H.: Special Instrumentation Problems Encountered in Physiological Research Concerning the Heart and Circulation in Man, Science **112**: 707-715, 1950.
9. Isaacson, James, and Jones, R. E.: An Electromagnetic Hydraulic Transducer for Studying Response Characteristics of Manometer Systems to Square Wave and Sine Wave Pressure Variations of Variable Frequency (Abstr.), Am. J. Physiol. **163**: 722-723, 1950.
10. Jones, R. E.: Design and Reciprocity Calibration of a Hydrodynamic Pistophone, Thesis, Graduate School, University of Minnesota, 1952.
11. Lilly, J. C., Legallais, Victor, and Cherry, Ruth: A Variable Capacitor for Measurements of Pressure and Mechanical Displacements: A Theoretical Analysis and Its Experimental Evaluation, J. Appl. Physics. **18**: 613-628, 1947.
12. Wood, E. H., Leusen, I. R., Warner, H. R., and Wright, J. L.: Measurement of Pressures by Cardiac Catheters in Man, Minnesota Med. **37**: 87-92, 1954.
13. Wood, E. H.: Physical Response Requirements of Pressure Transducers for the Reproduction of Physiological Phenomena, Tr. Am. Inst. Electrical Engineering. (In press.)
14. Hansen, A. T.: Pressure Measurement in Human Organism, Acta physiol. scandinav. **19** (suppl. 68): 1-230, 1949.

# A RAPID PROCEDURE FOR THE DETERMINATION OF LEAD IN BLOOD OR URINE IN THE PRESENCE OF ORGANIC CHELATING AGENTS

SAMUEL P. BESSMAN, M.D., AND ENNIS C. LAYNE, JR., M.S.
WASHINGTON, D. C.

THE clinical diagnosis and study of lead poisoning has long been hampered by the difficulty of determining the quantity of lead present in urine or blood. With the advent of a promising method of therapy[1] this problem has become acute. Many opportunities for fruitful observation are lost because only a few laboratories can perform the analysis. The procedure to be reported is based on the dithizone-colorimetric method,[2] and represents a number of modifications designed to shorten the time required, lessen the danger of contamination, and diminish the large quantity of blood or urine usually necessary.

The dithizone method for lead determination utilizes the fact that lead forms a red chloroform soluble, complex with diphenylthiocarbazone (dithizone).[3] Although other metals, notably copper and iron, interfere, they can be eliminated by extraction of the lead dithizone complex from alkaline solution in the presence of cyanide. The excess dithizone is removed with ammonium hydroxide-cyanide solution, and the color complex remaining in the chloroform layer is then read in a colorimeter at an appropriate wave length, the red color density being proportional to the amount of lead in the initial sample. One of the major difficulties which besets all methods for lead is the presence of significant quantities of lead in ordinary distilled water, necessitating the use of glass redistilled water. This problem has been, for the most part, eliminated through the use of a resin demineralizer to purify the distilled water as needed. The demineralizer (Deminac, A. S. Aloe) we use is a commercial unit consisting of a 500 ml. polyethylene bottle, in the neck of which is fixed a cylinder containing both cationic and anionic resins. Ordinary hospital distilled water, containing significant quantities of lead, is adequately deleaded merely by forcing the water to filter through the resin tube under pressure generated by squeezing the bottle. All glassware is given a final rinse with this water and all reagent solutions are made up with it. The entire determination, up to the colorimetry, is performed in the same glass-stoppered Nessler-type tube, eliminating the separatory funnels usually required.

## APPARATUS

A glass-stoppered Folin-Nessler tube is used to perform the entire determination. This can be made in the laboratory by attaching a standard taper 24/40 female joint to the regular 25 by 200 mm. digestion tube. The total length of this tube is 260 mm. These are stoppered by the standard glass stopper. (Fig. 1.) Glass beads previously are washed in detergent, then in lead-free water, then in glacial acetic acid, and then in lead-free water.

From the Research Foundation of Children's Hospital, Washington, D. C.
Aided by a grant from the Playtex Park Foundation.
Presented in part at the May, 1954, meeting of the Society for Pediatric Research.
Received for publication July 29, 1954.

## REAGENTS

*Digestion Reagent.*—Five parts nitric acid plus two parts sulfuric acid v/v.

*Buffer Solution* (Modified from Amdur[4]).—One hundred eighteen grams dibasic ammonium citrate plus 75 ml. concentrated ammonium hydroxide diluted to 250 ml. When it is cooled to room temperature, add 5 Gm. KCN and 2.5 Gm. sodium sulfite. Extract with 30 mg. per liter dithizone in chloroform until the dithizone stays green. Add 500 ml. ammonium hydroxide. (This entire procedure is performed in a glass-stoppered Pyrex bottle on the outside of which are marked lines corresponding to volumes to which the solution is made. The extraction is accomplished by adding approximately 2 ml. aliquots of dithizone and shaking the stoppered bottle. The chloroform-dithizone solution is sucked off from beneath the aqueous layer by means of a water-pump and drawn-out glass tube, which are used in the subsequent method.)

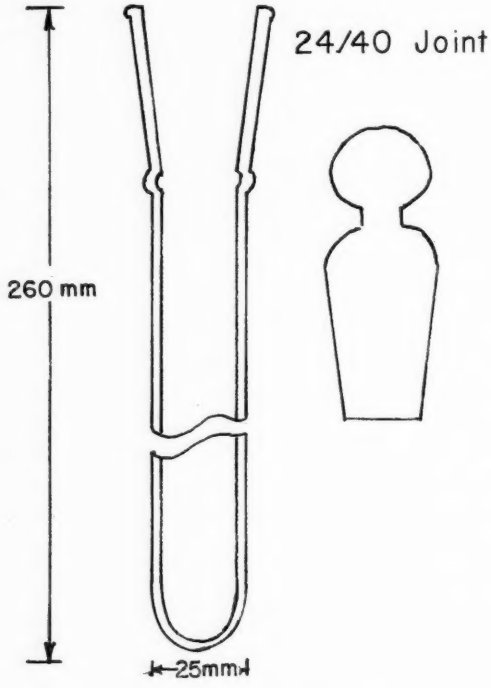



Fig. 1.—Digestion-extraction tube.

*Wash Reagent.*—Five grams KCN plus 250 ml. $NH_4OH$ are diluted to 500 ml. (This solution is not critical and can be made up in a glass-stoppered bottle with 250 and 500 ml. marks drawn on it with a China marking pencil.)

*Phenol Red.*—One-tenth per cent.

*Perchloric Acid.*—C. P. 70 per cent.

*Lead Standard.*—Lead acetate trihydrate 36.6 mg. per liter. (As a working standard we dilute this solution 1 to 10 and use 2 and 4 ml. aliquots corresponding to 4 and 8 γ of lead.)

*Dithizone Solution.*—Thirty milligrams dithizone, Eastman, per liter of chloroform.

*$NH_4OH$,* C. P.

## METHOD

1. Place samples in digestion tubes, 2 ml. blood, or 10 ml. urine.

2. Add 5 ml. digestion reagent and three or four glass beads.

3. Digest over a microburner until tube is filled with thin, white fumes. Take away burner.

4. Add 20 drops perchloric acid.

5. Heat again until the solution goes through the following changes: It becomes yellow, then colorless, then yellow again with frothing, and finally colorless and clear. (Blood may remain very faintly yellow.) At this point the digestion is completed. At times the solution will turn colorless, particularly with urine, when the perchloric acid is added. It must, nevertheless, be heated through the yellow frothing phase to the colorless stage in order to complete the digestion.

6. Cool. Add about 5 ml. water, swirl, and add 4 ml. $NH_4OH$ and 1 drop of phenol red. When this mixture is swirled (cool) the phenol red must remain red, indicating that the solution has been neutralized sufficiently. If the proper alkalinity is not attained, add $NH_4OH$ dropwise till the indicator is red.

7. To the cool solution add 10 ml. buffer solution and 3 ml. dithizone solution.

8. Stopper and shake vigorously twenty times in a longitudinal direction.

9. Take off supernatant by drawn-out pipette, using water-pump suction. At this point it is necessary to draw off most of the aqueous reagent; the critical point can be observed in this drawing off by watching for a ring of liquid which rises from the surface of the chloroform layer when there is no longer enough water over the chloroform phase to maintain an intact water layer. This ring of water rises several millimeters up the side of the tube and indicates plainly when sufficient aqueous phase has been sucked off.

10. Add 10 ml. wash reagent and shake again until clear.

11. Take off the supernatant by suction, again watching for the sign of sufficient removal of the aqueous layer.

12. Pour the chloroform solution into a colorimeter tube 1 cm. in diameter, stopper it with a cork, and allow to stand for ten minutes to clear. The chloroform solution can also be centrifuged gently if a more rapid clearing is desired. Too rapid centrifugation will cause clouding.

13. Read the chloroform layer at 510 $m\mu$ against a blank of chloroform.

The optical density of the solution after reagent blanks which have been run through the procedure are subtracted is directly proportional to the amount of lead in the original sample. The amount of dithizone present in the aliquot of buffer solution is sufficient to account for all the lead which might be encountered in urines containing up to 1,500 $\gamma$ per liter of lead. However, the optical density at this level is unreadable. These deeply colored solutions can be diluted with chloroform and read without error. The curve is linear over the range of 1 to 10 $\gamma$ of lead per specimen (Fig. 2), corresponding in concentration at the suggested amounts of blood or urine taken to from 50 to 500 $\gamma$ per cent of blood lead or to from 100 to 1,000 $\gamma$ per liter of urine.

If, at Step 8, the dithizone layer is colored red, an additional 3 ml. of dithizone solution should be added, and the mixture shaken again. Follow through with the rest of the method and double the value read to get the correct value for total lead in the specimen. The blanks for this method are quite consistent for any set of reagents, and duplicates are run with each set of twenty-four tubes. The total blank is equivalent to approximately 1 $\gamma$ of lead. Twenty-four tubes furnish ten determinations in duplicate plus a pair of standards and duplicate blanks. Such a set of tubes can be tested in less than three hours, including setting up the whole group from the original specimens. A single determination, including duplicate standards, blanks, and unknowns, can be run in less than one hour. The above times are regularly obtained when a battery of eight microburners is utilized for the digestion.

Duplicate determinations of forty-two consecutive urine specimens showed a mean difference of 2.16 ± 1.99 per cent from the mean of any pair. Twenty-six consecutive whole-blood determinations showed a mean difference of 3.70 ± 2.04 per cent from the mean of any pair.

In the determination of blood, it is necessary at Step 7 to add the chloroform dithizone solution and extract immediately without allowing the mixture to stand for more than a minute or two. Otherwise, the chloroform layer becomes colored yellow and the lead dithizonate is not extracted. If two tubes are mixed at the same time at this point, there is no difficulty with this yellow color developing, but if a series of tubes is first treated with buffer and then extracted with the dithizone reagent, a yellow color frequently appears, rendering the determination useless. This is probably due to a complex of iron cyanide and dithizone. The same phenomenon does not occur in urine, and urine can be treated in any way with any time length between the addition of reagents without difficulties arising.

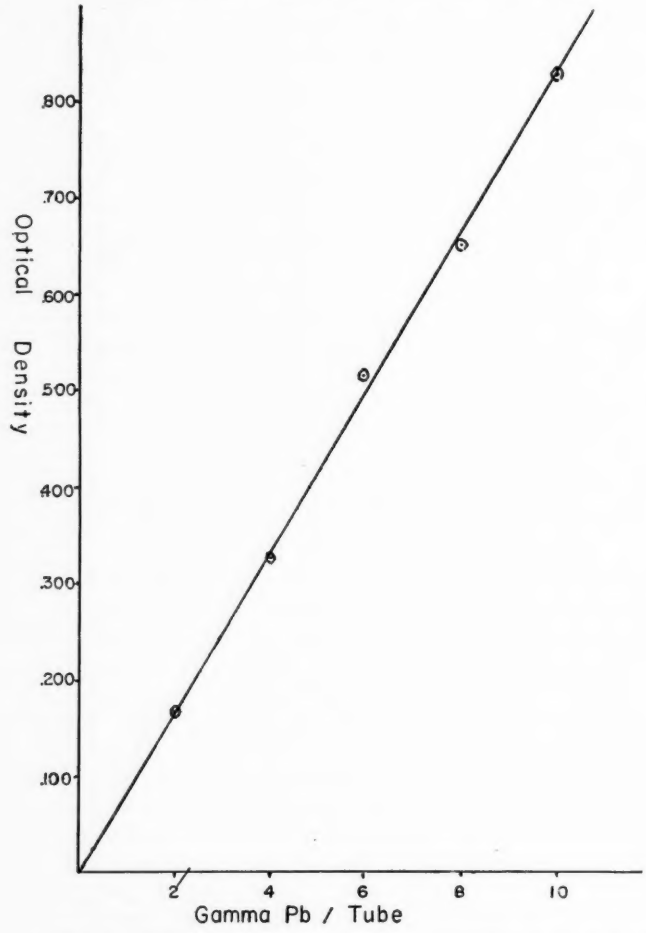



Fig. 2.—Color density in relation to concentration of lead.

## RESULTS

Fig. 2 shows that the dithizone lead color developed by this method is proportional to the lead taken in the original sample. Table I gives the recovery of lead added to standards, and to blood and urine. Table II shows

TABLE I. RECOVERY OF LEAD ADDED TO BLOOD AND URINE*

| SPECIMEN | LEAD ADDED | LEAD FOUND | % OF THEORETICAL |
|---|---|---|---|
| Urine 8/18 | | 0.35 | |
| | 4.0 | 4.10 | 94 |
| Urine A.J. | | 2.65 | |
| | 4.0 | 6.29 | 95 |
| Urine J.C. | | 2.00 | |
| | 4.0 | 5.71 | 95 |
| Blood (1) | | 0.71 | |
| | 4.0 | 4.90 | 102 |
| Blood (2) | | 0.74 | |
| | 4.0 | 4.38 | 92 |

*Values for lead expressed in gamma.

TABLE II. RECOVERY OF LEAD IN THE PRESENCE OF CALCIUM DISODIUM ETHYLENE DIAMINE TETRAACETATE (CaEDTA)*

| CaEDTA ADDED (MG.) | LEAD ADDED | LEAD FOUND | % THEORETICAL |
|---|---|---|---|
| 25 | 4.0 | 3.71 | 93 |
| 50 | 4.0 | 4.19 | 105 |
| 100 | 4.0 | 2.86 | 72 |

*Values for lead expressed in gamma.

the effect of calcium ethylene diamine tetraacetate on the determination of lead by this method. Varying amounts of a solution of the chelating agent prepared according to our first publication[1] were added to the blood or urine specimens prior to digestion. Table III gives the data for duplicate determinations on twenty-six consecutive specimens of blood and forty-one urine specimens from patients with lead poisoning at various stages of therapy. The amount of lead found in the first and second sample of each pair is given under the headings "gamma (1)" and "gamma (2)," and the difference in gamma of lead is shown in the next column. The difference between duplicates as a per cent of the mean of the pair is given in the last column. At the end of the group of blood values and the group of urine values are the mean and standard deviations of both the absolute differences in gamma and the per cent differences from the means. The determination marked with an asterisk was omitted from the calculation of the mean for that group because it differed from the mean by more than three standard deviations. All values greater than 10 gamma of lead were read after dilution of the colored solution appropriately with chloroform.

Twelve determinations on the blood of patients with no history of exposure to lead gave a mean of 18.2 ± 11.2 $\gamma$ per 100 ml., with a range of 0 to 32 $\gamma$.

## DISCUSSION

The use of a single tube for the digestion and extraction obviates the need for quantitative transfers of the test solutions at any point before the excess dithizone has been removed. At the point of removal there is no possibility of accidentally contaminating the chloroform extract, since there is no unreacted dithizone left.

TABLE III. DUPLICATE DETERMINATIONS ON BLOOD AND URINE

| SPECIMEN | GAMMA (1) | GAMMA (2) | DIFFERENCE | % OF MEAN |
|---|---|---|---|---|
| | | *Blood* | | |
| G4/7 | 3.57 | 3.47 | 0.10 | 2.8 |
| G4/8 | 4.55 | 4.43 | 0.12 | 2.7 |
| G4/9 | 2.59 | 2.55 | 0.04 | 1.6 |
| G4/11 | 1.33 | 1.45 | 0.12 | 8.6 |
| G4/12 | 1.06 | 1.25 | 0.21 | 17.9 |
| G4/10 | 1.52 | 1.67 | 0.15 | 9.4 |
| G4/13 | 1.44 | 1.58 | 0.14 | 9.3 |
| S9/4 | 0.90 | 0.83 | 0.07 | 8.0 |
| S2/h | 0.60 | 0.56 | 0.04 | 6.9 |
| S4h | 0.48 | 0.52 | 0.04 | 8.0 |
| S6h | 1.02 | 0.98 | 0.04 | 4.0 |
| D8/20 | 1.03 | 1.13 | 0.10 | 9.3 |
| D8/21 | 1.33 | 1.25 | 0.08 | 6.2 |
| D8/22 | 1.15 | 1.05 | 0.10 | 9.1 |
| D8/23 | 0.85 | 0.92 | 0.07 | 7.9 |
| D8/24 | 0.82 | 0.88 | 0.06 | 7.1 |
| J | 6.75 | 6.42 | 0.33 | 5.0 |
| R5/16 | 0.85 | 0.85 | 0.00 | 0.0 |
| R5/17 | 1.23 | 1.15 | 0.08 | 6.7 |
| R5/19 | 1.27 | 1.35 | 0.08 | 6.1 |
| R5/20 | 0.87 | 0.77 | 0.10 | 12.2 |
| P6/15 | 1.35 | 1.31 | 0.04 | 3.0 |
| W0 | 0.62 | 0.68 | 0.06 | 9.1 |
| W2 | 0.92 | 1.00 | 0.08 | 8.3 |
| W4 | 0.66 | 0.62 | 0.04 | 6.3 |
| W6 | 0.55 | 0.66 | 0.11 | 18.1 |
| Mean | | | 0.092 ± 0.063 | 7.45 ± 4.20 |
| | | *Urine* | | |
| S8/25 | 1.16 | 1.10 | 0.06 | 5.3 |
| D8/20 | 1.97 | 1.89 | 0.08 | 4.1 |
| S | 0.53 | 0 56 | 0.03 | 5.5 |
| D8/21 | 7.07 | 7.47 | 0.40 | 5.5 |
| D8/24 | 15.72 | 16.86 | 1.14 | 7.0 |
| D8/25 | 14.59 | 14.02 | 0.57 | 4.0 |
| D8/26 | 5.20 | 5.50 | 0.30 | 5.6 |
| D8/27 | 9.63 | 9.60 | 0.03 | 0.3 |
| S | 5.14 | 5.06 | 0.08 | 1.4 |
| 20-21 | 3.48 | 3.53 | 0.05 | 1.6 |
| 21-22 | 12.36 | 12.99 | 0.63 | 5.0 |
| 22-23 | 16.86 | 17.50 | 0.64 | 3.7 |
| 23-24 | 32.90 | 30.74 | 2.16 | 6.8 |
| 24-25 | 21.64 | 20.58 | 1.06 | 5.0 |
| 25-26 | 9.75 | 10.04 | 0.29 | 2.9 |
| 28-29 | 1.39 | 1.36 | 0.03 | 2.2 |
| 29-30 | 22.76 | 23.32 | 0.56 | 2.4 |
| 30-31 | 7.49 | 7.99 | 0.50 | 6.5 |
| 1-2 | 9.26 | 9.89 | 0.63 | 6.6 |
| 6/30-7/1 | 4.28 | 4.25 | 0.03 | 0.8 |
| 7/1-7/2 | 14.92 | 15.47 | 0.55 | 3.6 |
| 7/2-7/3 | 11.24 | 11.12 | 0.12 | 1.1 |
| 7/3-7/4 | 5.35 | 5.50 | 0.15 | 2.8 |
| D29/30 | 5.47 | 5.33 | 0.14 | 2.6 |
| D30-31 | 2.87 | 3.00 | 0.13 | 4.5 |
| D31-9/1 | 3.64 | 3.64 | 0.00 | 0.0 |
| D1-2 | 3.34 | 3.38 | 0.04 | 1.2 |
| D2-3 | 2.83 | 2.94 | 0.11 | 3.8 |
| D3-4 | 0.91 | 0.86 | 0.05 | 5.6 |
| S | 2.33 | 2.35 | 0.02 | 0.9 |
| S | 1.63 | 1.43 | 0.20 | 13.1 |
| S | 2.12 | 2.29 | 0.17 | 7.7 |
| S | 2.29 | 2.12 | 0.17 | 7.7 |
| 8/2-3 | 2.43 | 2.53 | 0.10 | 4.0 |

TABLE III—CONT'D

| SPECIMEN | GAMMA (1) | GAMMA (2) | DIFFERENCE | % OF MEAN |
|---|---|---|---|---|
| 8/3-4 | 13.38 | 13.28 | 0.10 | 0.8 |
| 7/13-14 | 21.08 | 21.50 | 0.42 | 1.9 |
| 7/14-15 | 7.40 | 7.61 | 0.21 | 2.8 |
| 7/16-17 | 9.14 | 11.50 | 2.36* | 22.9* |
| 7/17-18 | 15.49 | 16.05 | 0.56 | 3.6 |
| 7/18-19 | 5.52 | 5.49 | 0.03 | 0.6 |
| 6/28-29 | 9.24 | 9.53 | 0.29 | 3.1 |
| 6/29-30 | 7.89 | 8.29 | 0.40 | 4.9 |
| Mean | | | $0.323 \pm 0.407$ | $3.86 \pm 2.61$ |

*Omitted from the calculation of the mean for this group because it differed from the mean by more than three standard deviations.

The use of the deionizer has the advantage of furnishing an unlimited supply of lead-free water and of eliminating the need for setting up and maintaining a glass distillation apparatus. The small samples required permit digestion in a relatively short period, and the volumes employed are much easier to handle than the 10 ml. of blood and 25 ml. of urine required for previous methods. The adjustment of the amount of dithizone to include a wide range of possible values, due to the small samples originally taken, permits the extraction to be carried out usually with one aliquot of the dithizone solution. This procedure eliminates the need for multiple transfers.

If bismuth is present in the sample, it can be removed at the neutralization step (Step 6) by bringing the pH of the digestion solution to about 3.4 (indicator paper) with the concentrated ammonia, and extracting with 5.0 ml. of dithizone solution. Discard the dithizone layer completely, using the drawn-out pipette and suction. Add the phenol red, alkalinize with ammonia, and continue according to the above method.

The recovery of added lead tends to be low (about 5 per cent in urine), and longer digestion does not increase the yields. We have not considered this serious enough to investigate it extensively.

The blood levels of patients who have not been exposed to lead fall within the normal range[5] as do the urine levels.

A few precautions should be emphasized, and when they are taken, there should be no difficulty in using this method in a routine laboratory:

1. The glassware must all be cleaned with a detergent solution, rinsed with ordinary distilled water, and then rinsed thoroughly with deionized water.
2. All solutions should be made up with deionized water.
3. The digestion tube should be kept covered with a 50 ml. beaker at all times except during the initial evaporations.
4. The tube should be permitted to cool at least one minute before the addition of perchloric acid.
5. Blood determinations should not be permitted to stand with the buffer reagent more than two minutes before shaking with dithizone.

## SUMMARY

A simplified procedure for the determination of lead in small samples of urine and blood by the dithizone method is reported. The entire determina-

tion is performed in a glass-stoppered digestion tube. Resin deionized water is used in place of glass redistilled water. The agreement between duplicate determinations on urine is 3.86 ± 2.61 per cent, and between duplicate blood levels is 7.45 ± 4.20 per cent. Calcium ethylene diamine tetraacetate at levels equivalent to 500 mg. per cent in urine and 2,500 mg. per cent in blood does not interfere.

The authors wish to thank the Riker Laboratories for a supply of Disodium Calcium Versenate.

REFERENCES

1. Bessman, S. P., Ried, H., and Rubin, M.: Treatment of Lead Encephalopathy With Calcium Disodium Versenate, Med. Ann. Dist. Columbia **21**: 312, 1952.
Belknap, E. L.: EDTA in the Treatment of Lead Poisoning, Indust. Med. **21**: 306, 1952.
2. Bambach, K., and Burkey, R. E.: Microdetermination of Lead by Dithizone With an Improved Lead-Bismuth Separation, J. Indust. and Eng. Chem., Anal. Ed. **14**: 904, 1942.
3. Fischer, H.: Uber den Nachweis von Schwermetallen mit Hilfe von "Dithizon," Ztschr. Angewandte Chemie **42**: 1025, 1929.
4. Amdur, M. O.: Rapid Determination of Lead in Old Urine Samples, A. M. A. Arch. Indust. Hyg. **7**: 277, 1953.
5. Kehoe, R. A.: Report of Committee on Lead Poisoning, Pub. Health Rep., New York, 1943.